RAPPORT 2004
VERSLAG

La Reine Elisabeth à La Panne devant l'hôtel l'Océan transformé en hôpital

Koningin Elisabeth bij het als hospitaal ingerichte hotel l'Océan in De Panne

Fondation Médicale Reine Elisabeth

Geneeskundige Stichting Koningin Elisabeth

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verslag rapport

2004

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2004

Inleiding
Verslag Activiteiten van de GSKE – FMRE

Ondertussen is er al 3 jaar verlopen sinds de laatste projectselectie. De weerhouden ploegen hebben voor een totaal bedrag van 3 miljoen EURO aan kredieten kunnen gebruiken.
Het is dan ook met een groot genoegen dat ik U het rapport kan voorstellen die de onderzoeken van de laatste 3 jaar verzamelt

De gerealiseerde vooruitgang in de verschillende projecten werden gepubliceerd in gerenommeerde wetenschappelijke tijdschriften. Dit is een bewijs van de kwaliteit en het belang van de gerealiseerde werken.
Niet minder dan 232 artikels zijn gepubliceerd gedurende de 3 laatste jaren met de vermelding van de G.S.K.E.
De hergroepering van de verschillende onderzoekslaboratoria in het domein van de Neurowetenschappen in België, heeft er toe geleid dat de G.S.K.E. beschouwd wordt als de pilootinstelling in deze discipline.
Bij deze gelegenheid wens ik de Raad van Bestuur te bedanken voor hun constante dynamische en constructieve stijl waarmee de voorstellen van het Wetenschappelijk Comité behandeld worden.

Onze dank gaat vooral naar H.K.H Prinses Astrid. Haar permanente zorg voor ieders welzijn en haar wilskracht om zich persoonlijk en volledig in te zetten tijdens de door Haar geleide zendingen, betekent een troost voor degene die Zij ontmoet.
Haar aanwezigheid in de verschillende laboratoria staat altijd garant voor interesse en aanmoediging voor hen die hun leven wijden aan deze dikwijls moeilijke, onzekere en soms tijdrovende roeping.

De vergadering van het Wetenschappelijk Comité op 11 december 2004 heeft geleid tot een rangschikking van de ingezonden projecten.De voorstellen zijn begin 2005 voorgelegd aan de Raad van Bestuur.

Prof. Dr. Th. de Barsy
Wetenschappelijk Directeur
Brussel, maart 2005

Fondation Médicale Reine Elisabeth

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Introduction
Rapport d'Activités de la FMRE - GSKE

Trois ans se sont déjà écoulés depuis dernière sélection de projets de recherche. Les équipes retenues ont pu bénéficier de crédits pour une somme globale de 3 million d'euro.
C'est avec plaisir et fierté que je puis vous présenter le rapport rassemblant les recherches de cette dernière année.

Les progrès réalisés dans les différents projets ont pu être rapportés dans des revues de haut niveau scientifique, témoignant de la qualité et de l'importance des travaux réalisés.
Au cours des ces 3 dernières années 232 articles ont pu être publiés avec la mention de l'aide accordée par la FMRE.
Le regroupement de plusieurs laboratoires consacrés à la recherche dans le domaine des neurosciences en Belgique a permis de placer la FMRE comme institution pilote dans cette discipline.
C'est l'occasion de remercier les membres du conseil d'administration pour son dynamisme et son soutien constant en répondant de façon constructive aux propositions du comité scientifique.

Nos remerciements vont tout particulièrement à S.A.S la Princesse Astrid, qui,par son attention, son souci permanent du bien de chacun, sa volonté de s'investir personnellement et pleinement dans les missions dont elle a accepté la charge, apporte un réconfort chaleureux à tous ceux et celles qu'elle rencontre. Sa présence, dans les différents laboratoires qu'elle a pu visiter, a toujours été un gage d'intérêt et un signe d'encouragement pour ces hommes et femmes qui consacrent leur vie à cette vocation souvent difficile, austère et pleine d'incertitude.

La réunion du comité scientifique du 11 décembre 2004 a permis de dégager un classement des projets soumis pour une nouvelle durée de trois ans.Les propositions seront présentées au conseil d'administration en début d'année 2005

Prof. Dr. Th. de Barsy
Directeur Scientifique
Bruxelles, mars 2005

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Final Reports of the University Research Groups, supported by the Queen Elisabeth Medical Fondation in collaboration with the following Professors and Doctors (2002-2004)

Final Report of the Research Group of

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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

During development, neural cells extend processes that are guided to their destination by short and long range repulsive or attractive guidance cues (Dormann and Weijer, 2003). Formation of these processes is critically dependent on polymerisation and dynamic turnover 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 Gallo and Letourneau 2004). An emerging picture from the last years is that the balance of actin polymerisation/depolymerisation 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 thymosinβ -members, each modulate a different point of the actin polymerisation cycle (Lambrechts et al., 2004). 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 (Pollard and Borisy, 2003). 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., 2000a) suggests an important role for the interaction of these proteins at this stage of neuronal development. EVL, an Ena/VASP-family member, nucleates actin polymerisation *in vitro* (Lambrechts et al., 2000b) and profilins promote actin filament elongation if free polymerising filament ends are available (Pantaloni and Carlier, 1993; Lambrechts et al., 2000a).

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 family members. With the exception of the latter family all actin binding proteins are regulated by a variety of signal transduction mechanisms. For instance EVL is regulated by Protein kinase A and possibly by interaction with n-Src kinase and FE65 (Lambrechts et al., 2000b) and the phosphoinositol-4,5 -bisphosphate controls activity of profilins and cofilins (Ampe and Vandekerckhove, 2005).

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 studies 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

Model systems and assays.

We employ several model systems: 1) 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], 2) NG108-15 and CAD cells which larger fan shaped growth cones than the PC-12 cells, 3) primary murine hippocampal neurons and 4) the model organism C. *elegans*. We have developed an assay system to measure number and length of neurites formed by an inducible expression system in PC-12. This was 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. We have set up a live-video microscopy system to monitor neurite formation over longer periods of time.

C. elegans actin PFN1 and tetraThymosin β binding proteins are present in the nerve ring during embryogenesis at stages requiring active actin remodelling.

C. elegans profilin I (PFN-1) is expressed in the neuronal ring during development (Polet et al. in preparation). Similarly tetraThymosin β is expressed in this organ (Van Troys et al., 2004). Their expression in this organ is consistent with the dynamic actin reorganization required at this stage. Silencing of Profilin 1 expression results in a cell division defect and embryos die at a very early stage (Severson et al. 2003). The knock-out of tetraThymosin β in C. *elegans* results in a lethal "dumpy " phenotype in early adults (Van Troys et al., 2004). Profilins and tetrathymosin detection requires special fixation conditions. Therefore to further analyse expression we isolated the promotor regions of the respective genes and fused it to GFP (Polet, Van Troys et al., unpublished). These constructs are currently used to further probe tissue specific expression of these proteins, eventually also enabling to monitor developing neurons.

Given that UNC-34 was shown to function both in axonal attraction, as well as axon repulsion, in the netrin receptor UNC140-40/DCC pathway (Gitai et al., 2003). We cloned the two splice variants of unc-34 (the C. elegans homologue of Mena, see below). We expressed domains of the proteins and raised antibodies against these proteins. Immunostaining of the two Unc splice variants revealed a different staining for the two proteins in worm (Polet, Ono et al., unpublished).

Different profilin I activities are required for proper neurite formation in PC12 cells.

Profilins display several activities: actin binding phosphoinositide binding and interaction with proline-rich sequences such as occurring in Ena/VASP-members. Mammals have four profilin isoforms of which two: profilin I and IIa, are expressed in neuronal tissues (Lambrechts et al., 2000a). 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). We constructed various profilin I mutants aimed at disrupting each of the three activities. However our results showed that 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) complicating interpretation of results. The profilin I mutants R74E (only defective in actin binding) and R136D (defective

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

in PIP_2 and polyproline binding) have been biochemically characterized (Lambrechts et al., 2002). In addition, we constructed and characterized a profilin I W3A mutant defective in polyproline binding (Lambrechts et al., unpublished). Stably transfected PC12-cell lines expressing similar levels of WT and mutants allowed comparing the effects of the mutants on neurite extension upon NGF/FS stimulation. For each mutant we scored number of neurites per cell, length of neurites and number of branches, five days after stimulation with NGF/FS and compared it with WT (moderate overexpression of WT profilin does not result in a phenotype, significantly different from the parental cell line) Of interest is that each of the mutants, defective in one or two activities causes a different phenotype. Although R74E showed little effect on neuritogenesis in PC12-cells (still containing endogenous WT-profilin), additional silencing of WT profilin resulted in lethality likely due to a cell division defect. In cell populations expressing mutant R136D more cells have more and longer neurites and more branches compared to the NGF induced parental PC12-cell line (or to WT). In contrast, profilin I W3A has shorter neurites with more branches. These results suggest that PIP_2-binding by profilin, or the balance of the interaction between polyphosphoinositides and proline-rich actin binding proteins, has an important contribution in neurite outgrowth. In addition, inhibition of Rho-kinase results in longer neuritis, except in profilin I R136D expressing cells (Lambrechts et al., in preparation). Since polyphosphoinositide metabolism occurs downstream of Rho-kinase this further points to an important role of the profilin phosphoinositol-4,5 -bisphosphate interaction in neurite formation. Given the results with profilin W3A it is know also important to pursue the Ena/VASP connection. Various EVL mutants with reduced or abolished profilin binding have been constructed (Veniere et al., unpublished) and will be shortly introduced in some of the PC12 cell lines. Our long-term goal here is to dissect the combined effect of EVL (or Mena) and profilin I on actin dynamics, and to understand their interplay in neuronal outgrowth.

Profilin IIa associates with the Survival of Motor Neuron Protein a protein involved in β-actin mRNA transport

Profilin IIa is the most abundant form in neurons (Lambrechts et al., 2000a). We could recently show that profilin IIa is a binding partner for SMN (Survival Motor Neuron protein) a protein implicated in spinal muscle atrophy, a disease characterized by degeneration of motoneurons. Pathogenic missense mutations in SMN, or deletion of exons 5 and 7, prevented this interaction. SMN and profilin IIa colocalise in the cytoplasm of differentiating rat PC12 cells and in neurite-like extensions, especially at their leading edges. SMN is known to associate with specific hnRNPs in axons and neurites and SMN also stimulates neurite outgrowth in cultures. Antisense knockdown of profilin I and II isoforms inhibited neurite outgrowth of PC12 cells and caused accumulation of SMN in cytoplasmic aggregates, suggesting that profilin-regulated growth of actin microfilaments is required for normal SMN distribution in neuronal cell cytoplasm. (Sharti, et al., submitted). The interaction is functional in that SMN can modulate actin polymerisation in vitro by reducing the inhibitory effect of profilin IIa. This suggests that reduced SMN in SMA might cause axonal path finding defects by disturbing the normal regulation of microfilament growth by profilins.

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(Papers marked with * have been realized within the framework of GSKE-projects)

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Final Report of the Research Group of

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In collaboration with

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

Our laboratory has many years of experience with research on the molecular mechanisms directly related to brain plasticity in higher mammals. We use the visual system of the cat as research model to study and compare two forms of brain plasticity, firstly the effect of small binocular retinal lesions in adult animals and secondly developmental brain plasticity. We envisage that this approach will help in understanding the (dis)-similarities in the molecular mechanisms underlying [1] the more reduced but nevertheless apparent capacity for cortical plasticity later in life and [2] the exceptional capacity for plasticity in the first critical period of visual system development.

The identification of new mediators of brain plasticity will ultimately lead to the elucidation of the underlying molecular cascade, which will boost the development of new pharmaceuticals useful in the treatments for sensory loss and brain damage and for goal-directed improvement of post-lesional recovery of brain function.

1) The role of the excitation-inhibition balance in adult cortical plasticity

1a) Glutamate levels and transport in cat visual cortex

Glutamate is known to play a crucial role in the processes of cortical reorganization after the induction of binocular central retinal lesions (Arckens et al., 2000). In this study we investigated the possible involvement of the glial high-affinity Na^+/K^+-dependent glutamate transporters using intracortical microdialysis and Western blotting. A direct measure for the re-uptake activity for glutamate has been provided by measuring the increase in extracellular glutamate concentration upon blocking its removal from the synaptic cleft with the potent transporter inhibitor L-trans-pyrrolidine-3,4-dicarboxylic acid. In cats with central retinal lesions we measured increased basal extracellular glutamate concentrations in peripheral area 17, far outside the lesion-projection zone (LPZ) together with a decreased re-uptake activity, and this compared to the LPZ of the same animal as well as to the topographically matching regions of area 17 in normal subjects. We further detected a parallel decrease in the expression level of the glial glutamate transporter proteins GLAST and GLT-1 outside the LPZ as compared to the LPZ and also to the corresponding regions of area 17 of normal subjects. This study shows that partial sensory deprivation of the visual cortex affects the removal of glutamate from the synaptic cleft and implicates a role for glial-neuronal interactions in adult brain plasticity.

Qu et al., Brain Res. 962:199-206, 2003

Massie et al., J. Neurochem. 84:1387-1397, 2003

1b) 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 LPZ of area 17, whereas three months post-lesion, normal GABAergic control is restored within the cortical scotoma (Rosier et al., 1995). In this study we used in vivo microdialysis to investigate the extracellular GABA concentrations one to two months post-lesion, in the LPZ 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 (see point 1a). As for glutamate, we observed significantly increased extracellular GABA concentrations in remote, non-deprived area 17, whereas in the LPZ of 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 LPZ 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 hyper-excitability is balanced by the moderately increased GABAergic control.

Massie et al., Brain Res., 976:100-108, 2003

1c) Distribution of the AMPA2 glutamate receptor subunit in cat visual cortex

To understand the effect of retinal lesions on the different subunits of the glutamate receptors we need a clear understanding of the distribution of all receptor subunits under normal visual conditions. In this study, we revealed the distribution of the AMPA2 glutamate receptor subunit in the visual cortical areas 17 and 18 of the adult cats. In situ hybridisation, using a cat-specific radio-active labelled oligonucleotide 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.

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

1d) Alterations in the subunit composition of glutamate receptors: an intriguing aspect of cortical topographic map plasticity

Long-term synaptic strengthening, one mechanism implicated in adult cortical plasticity, is supported by altered NMDA and AMPA glutamate receptor functioning. We used semi-quantitative Western blotting to determine changes in the protein expression level for five glutamate receptor subunits, AMPA1, AMPA2, NR1, NR2A and NR2B, in area 17 of adult cats in response to the induction of central binocular retinal lesions as a function of post-lesion survival time. We compared the expression levels for each of the subunits between the LPZ of area 17 and its peripheral, non-deprived counterpart. In comparison to normal controls, two weeks post-lesion we observed a significantly decreased AMPA1, AMPA2, NR1 and NR2B expression the LPZ of area 17. Most strikingly however, NR2A was increased up to three times. One month after the induction of the retinal lesions, NR2A dropped back to normal levels while at this stage in cortical reorganization NR2B showed a remarkable increase in the LPZ of area 17. These results connect subunit-specific and time-dependent fluctuations in the composition of ionotropic glutamate receptors to adult cortical plasticity.

2) Retinotopic map plasticity is accompanied by changes in Ca^{2+} / calmodulin-dependent protein kinase II alpha autophosphorylation

To investigate the possible involvement of the -subunit of the calcium/calmodulin dependent protein kinase type II (αCaMKII) in brain plasticity, we performed *in situ* hybridisation and Western blotting experiments to analyze mRNA, protein and autophosphorylation levels of this multifunctional kinase in primary visual cortex of cats with or without retinal lesions. No differences in the mRNA or protein levels were observed inside and outside the LPZ of area 17 in retinal lesion animals or between corresponding cortical regions of normal control animals. Nevertheless, Western blotting with an αCaMKII threonine-286 phosphorylation-state specific antiserum showed a 50 % higher level of αCaMKII autophosphorylation in the central versus the peripheral region of cortical area 17, and this both in normal subjects as well as in retinal lesion animals with a 3-day post-lesion survival time. In contrast, a post-lesion survival time of 14 days resulted in a αCaMKII autophosphorylation level that was 4 times higher in the LPZ of area 17 than in the non-deprived cortical region. If this increased phosphorylation state would have been attributable to the decreased visual activity in these neurons, we would have expected to see a similar change in phosphorylated αCaMKII at shorter or longer survival times after the induction of the lesions or in the left visually-deprived visual cortex of animals in which the left optic tract and the corpus callosum were surgically cut. This time-dependent change in the phosphorylation state of αCaMKII upon retinal lesioning suggests a role for phosphorylated αCaMKII in adult cortical plasticity.

Van den Bergh et al., Neurosci. 120:133-142, 2003

3) Differential display implicates the 'house-keeping' gene cyclophilin A and the transcription factor MEF2A in adult cortical plasticity

We used differential mRNA display (DDRT-PCR) to compare gene expression patterns inside and outside the LPZ of primary visual cortex, 3-days after induction of central retinal lesions in adult cat. Systematic screening revealed a decrease in the mRNA encoding cyclophilin A in the LPZ. In situ hybridisation and competitive PCR confirmed the decreased cyclophilin A mRNA levels in the LPZ and extended this finding to longer post-lesion survival times as well. Western blotting and immunocytochemistry extended these data to the protein level. *In situ* hybridization and immunocytochemistry further demonstrated that cyclophilin A mRNA and protein are present in neurons. To exclude the possibility that differences in neuronal activity per se can induce alterations in cyclophilin A mRNA and protein expression, we analysed cyclophilin A expression in the dorsal lateral geniculate nucleus (dLGN) of retinally lesioned cats and in area 17 and the dLGN of isolated hemisphere cats. In these control experiments cyclophilin A mRNA and protein were distributed like in normal control subjects indicating that the decreased cyclophilin A levels, as observed in the LPZ of area 17 of retinal lesion cats, are not merely a reflection of changes in neuronal activity. Instead our findings identify cyclophilin A, classically considered a housekeeping gene, as a gene with a brain plasticity-related expression in the central nervous system.

Systematic differential mRNA display (DDRT) screening further revealed higher levels for the mRNA encoding the transcription factor MEF2A in the LPZ. Semi-quantitative PCR confirmed this dependency of *mef2A* mRNA expression on visual eccentricity in area 17 of animals with retinal lesions, in contrast to normal animals. Western blotting experiments extended these data to the protein level and to two other members of the MEF2 transcription factor family, i.e. MEF2C and MEF2D. Quantitative analysis of the Western blotting experiments further disclosed a post-lesion survival time-dependent change in expression for all three MEF2 family members. The lesion effect was maximal at three days and one month post-lesion, but only minor at two weeks post-lesion. Interestingly, complete removal of retinal input from primary visual cortex by surgery did not significantly alter the expression of the MEF2 transcription factors in sensory-deprived visual area 17, excluding a definite correlation between neuronal activity and MEF2A expression levels. Immuno-cytochemistry for MEF2A confirmed both qualitatively and quantitatively the Western blotting observations in all animal models and further demonstrated the presence of the MEF2A protein exclusively in the nuclei of neurons across all cortical layers. Together, our findings identified a brain plasticity-related expression pattern for the MEF2 transcription factor family in adult mammalian neocortex.

Arckens et al., Eur. J. Neurosci. 18:61-75, 2003

Leysen et al., Eur. J. Neurosci., 20:769-780, 2004

4) The molecular correlates of the critical period of cat visual cortex

4a) 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 like 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.

The second goal of the present study was the selective enrichment and identification of low-abundance proteins within the same model of developmental brain plasticity. 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 frist 2D-DIGE 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.

Van den Bergh et al., J. Neurochemistry, 85:193-205, 2003

Van den Bergh et al., Electrophoresis 24:1471-1481, 2003

4b) Distribution of Collapsin Response Mediator Proteins (CRMPs) in kitten and adult cat visual cortex

The CRMP family consists of five cytosolic phosphoproteins (CRMP1-5) that are involved in neuronal differentiation during the development of the nervous system. They have been implicated in axon guidance and growth cone collapse through their action in the signalling pathway of collapsin/semaphorin. We examined the distribution of the CRMPs throughout the visual cortex of kitten and adult cat by in situ hybridization. While CRMP3 could not be detected in the visual cortex, the other CRMPs showed a higher expression in the immature brain compared to the adult state. Western blotting allowed the quantification of the observed age-dependent differences in the expression of CRMP2, 4 and 5. Moreover, for CRMP2 we observed a number of development-dependent posttranslational modifications. We thus conclude that CRMPs might be important during the normal postnatal development of the visual cortex possibly for the fine-tuning of the specific connections in the brain.

In a second part of this study, we compared in detail the CRMP2 and CRMP4 expression in developing (P10, P30), juvenile and adult cat primary visual cortex. Real-time PCR and semi-quantitative Western blotting assays clearly showed that the expression levels of CRMP2 and CRMP4 decreased with age and that this down-regulation was most pronounced for CRMP4. In addition, immunocytochemistry revealed that CRMP2 and CRMP4 exhibit an isoform-specific distribution in area 17. In adults, each CRMP is expressed in distinct cell types. Using quantitative and qualitative detection methods, we consistently showed that CRMP4 is less abundant in adult cat visual cortex than CRMP2 but nevertheless subsists significantly. CRMP2 and CRMP4 may therefore act complementary during visual cortex development and maturation. Furthermore, we believe that the presence of CRMP4 as observed in adult visual cortex under normal physiological conditions, cannot account alone for newly generated cortical neurons. Thus, CRMP4 cannot fulfill the role of an immature neuronal marker in adult mammalian neocortex but instead may be expressed by those neurons that maintain the intrinsic capacity to display axonal growth upon central nervous system lesions throughout life.

Cnops et al., Eur. J. Neurosci., 19:2345-2351, 2004

Cnops et al., Eur. J. Neurosci., submitted

5) The use of the immediate early gene (IEG) c-fos and its gene product Fos as markers for neuronal activity in cat visual cortex

5a) Molecular cloning and differential expression of the cat IEG c-fos upon sensory deafferentation

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 compare *c-fos* mRNA expression levels in central and peripheral regions of primary visual cortex in normal and retinal lesion cats.

Van der Gucht et al., Mol. Brain Res.,111:198-210, 2003

5b) Neurochemical characterization of Fos expressing neurons in cat visual cortex

Recent immunocytochemical stainings on cat visual cortex, visually stimulated for one hour, showed strongly induced Fos expression in cortical neurons (Van der Gucht et al., 2000). 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.

To determine which non-GABAergic neuronal populations exhibit Fos following an identical visual experience, we initiated double-stainings between Fos and phosphate-activated glutaminase (PAG) or Fos and neurofilament protein in cat area 17. These double-stainings revealed that Fos is also expressed in neurochemically distinct subpopulations of pyramidal neurons in supra- and infragranular layers of cat area 17.

Van der Gucht et al., Eur. J. Neurosci., 16: 1620-1626, 2002

Van der Gucht et al. (2005) Brain Res., in press

6) Distribution and morphological characterization of PAG-immunoreactive neurons in cat visual cortex

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.

Van der Gucht et al., Brain Res. 988:29-42, 2003

7) Exploration of the mouse visual system as a tool towards the analysis of true causal relationships between differential protein expression patterns and cortical plasticity - The characteristic expression of neurofilament protein defines six cortical areas and several subcortical divisions in mouse visual system.

It is now established that neurofilament protein has become a powerful tool for the parcellation of brain regions in different mammalian species. Nevertheless, there are no reports on mouse cortical organization based on a characteristic expression pattern of neurofilament protein. We therefore examined neurofilament protein staining profiles in mouse visual system as revealed with the SMI-32 monoclonal antibody. Neurofilament protein-positive neurons were prominent in six visual areas, but a remarkably reduced staining profile was present in the secondary visual areas. Topographic boundaries between adjacent areas could be delineated by a characteristic pattern of neurofilament protein such as differences in laminar distribution patterns, morphological and cellular characteristics and staining profiles. SMI-32 immunoreactivity was present in the dendrites and cell bodies of a subset of pyramidal neurons in three cortical layers III, V and VI. However, a minority of cortical layer II cells was also positive for neurofilament protein. We also found immunoreactivity in several subcortical structures. Although the dorsal and ventral lateral geniculate nucleus showed a strong immunolabeling, no cellular or fibrillary pattern was present in the intergeniculate leaflet. In addition, the laminar organization within the superior colliculus was clearly discernible based on differences in size and shape of positive cells and the dendritic arborization profile for neurofilament protein. The present study clearly shows that neurochemical subdivision of mouse visual system exists and is highly specific demonstrating organizational and functional

characteristics at the cellular and regional level. We are therefore convinced that the visual system of normal and retinal lesion mouse will be of great value as a model in future studies of cortical plasticity.

Van der Gucht et al., Soc Neurosci. Abstr, 2004

8) Technological advances in proteomics research - Sweet Substitute - A software tool for in silico fragmentation of peptide-linked N-glycans

Next to phosphorylation, also post-translational glycosylation of proteins is considered a potential mechanism of brain plasticity. We developed a software tool, Sweet Substitute, which assists MS/MS-based glycosylation characterization from within a tryptic digest. The algorithm creates a virtual nano-electrospray quadrupole time-of-flight style MS/MS spectrum of any user-defined N-linked glycan structure. An empirical peak height modeling routine is implemented in the program. By comparing the theoretical MS/MS data with the deconvoluted and deisotoped experimental MS/MS data, the user is able to quickly assess whether a proposed candidate oligosaccharide structure is a plausible one.

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Notch signaling during vertebrate early neural development.

The development of the nervous system requires the activation of a cascade of regulators involved in early patterning and neurogenesis. During the last three years, our research has focused on several genes involved in neural patterning and neurogenesis. Our laboratory showed that the XNAP/Nrarp gene coding for a small ankyrin repeat containing protein plays an important role in neurogenesis as overexpression of this gene in the Xenopus embryo increases the number of primary neurons that forms within the domains of the neural plate where they normally arise. We demonstrated that this gene is a direct target of the Notch pathway and that the XNAP/Nrarp protein stimulates neurogenesis by functioning, in a feedback loop, as a negative regulator of the Notch pathway (Lahaye et al., 2001; Pirot et al., 2004). We then isolated and characterized in Xenopus a novel member of the HRT/Hey subfamily, XHRT1, and showed that this factor plays an important role in floor plate development (Pichon et al., 2004; Pichon et al., 2002; Taelman et al., 2004).

During this last year sponsored by the Fondation Médicale Reine elisabeth, we concentrated on the mecanisms of action of the XSIP1/XZEB2 gene encoding a zinc finger transcriptional repressor expressed in neural precursor cells (van Grunsven et al., 2000) and have isolated another gene, DMRT5, encoding a DM type zinc finger gene with restricted expression in the developing anterior neural plate.

1. Functional analysis of the Xenopus XSIP1/ZEB2.

In collaboration with the laboratory of Prof. D. Huylebroeck, we compared Xenopus SIP1/ZEB2 and the related EF1/ZEB1 genes for their expression profile during Xenopus development and analyzed transcriptional repression capacities of both proteins. Furthermore we evaluated the potential of both proteins to bind to the co-repressors and co-activators described as interacting proteins of the Zfhx1 family (Smad, CtBP and PCAF) and used a classical domain-function approach to test SIP1's activity *in vivo* in Xenopus using Epidermal keratin repression as a read-out. Our results indicate that the two genes have distinct expression profile during embryogenesis but similar transcriptional repression potential. The interaction studies that have been conducted indicated that although the overall amino-acid similarity between SIP1/ZEB2 and δEF1/ZEB1 is not very high, the capacity of the two proteins to form complexes with co-activators and co-repressors is highly similar. In any case, the co-activators pCAF and p300 can form complexes with δEF1/ZEB1 and SIP1/ZEB2 leading -in the case of pCAF- to the release of CtBP from the ZEB proteins. Smad3 is present in SIP1/ZEB2 and δEF1/ZEB1 protein complexes but its presence requires p300 in the case of δEF1/ZEB1. In vivo domain function analysis of SIP1/ZEB2 showed that the zinc-finger clusters of SIP1/ZEB2 are necessary but not sufficient to repress transcription and that multiple regions of the protein are involved in transcriptional repression. Finally, we identified the INHAT protein NIR as a novel interaction partners for SIP1 and showed that it acts as a corepressor of SIP1/ZEB2 to transcriptionally regulate target genes during Xenopus development (van Grunsven et al., in preparation).

2. Identification and expression analysis of the DMRT5 gene.

The DMRT5 gene encode a member of a novel family of transcription factors containing a zinc finger-like DNA-binding motif first identified in the Drosophila doublesex (dsx) and Caenorhabditis mab-3 proteins (Hodgkin et al., 2002; Volff et al., 2003). We found that expression of this gene in Xenopus is first detected at early neurula stage in the anterior neural plate and that it is later restricted to the dorsal telencephalon and olfactory placodes. A similar highly restricted expression profile has been observed in the chicken and mouse embryos (V. Moers, unpublished data). We also analysed in gel shift assays the DNA binding properties of XDMRT5 and found that it binds to similar sequences as the mab3 and dsx proteins. Finally, using a two-hybrid approach, we identified the AES protein as a DMRT5 interacting partner. Further work is going on in our laboratory to characterize the function of DMRT5 in forebrain patterning in Xenopus by examining the effects of *XDMRT5* overexpression or inhibition of its translation using morpholino antisense oligonucleotides on the expression of various later markers of the forebrain and olfactory placodes (ex.: Xomp2,...). The importance of the DMRT5 gene in forebrain development will also be further investigated in the mouse system by generating *DMRT5* -/- mice.

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Experimental analysis of the microcircuitry of cerebellar cortex

Electrophysiological and morphological characterization of large interneurons of the granular layer

Whole-cell voltage-clamp recordings were obtained from microscopically identified putative Golgi cells in rat cerebellar slices. Biocytin injected cells were reconstructed using the Neurolucida computerised tracing system.

Golgi cells (n=10) were found in all depths of the granular layer. The cell bodies had rounded or polygonal shape and emitted 4 - 10 radiating dendrites. All cells had apical dendrites penetrating the molecular layer and basolateral dendrites restricted to the granular layer. The axonal plexus could be revealed in only three cases.

The cells could be subdivided into two groups. The majority of cells had short dendrites (n = 8). The distance along the dendrites from the soma to the most remote dendritic tip was: 327.2±78.7 m, whereas the maximal polar distance: 262.7±66.6 m. The average on path distance of all dendritic tips was: 159.9±52.2 m, and their average polar distance: 123.8±42.8 m. The ratio of the on path and polar distance (tortuosity factor) was 1.29±0.03, indicating a radial spread of dendrites. The average branch order of all dendritic segments was: 4.67±0.87 with a tortuosity factor of 1.29±0.03, indicating a radial spread of dendrites. Two out of ten cells had much longer dendritic trees with a maximal on path distance of 653.6 and 778.7μ m respectively. They branch order and tortuosity was however similar to that of the short dendritic neurons, indicating a similar topoloty.

Coding by Purkinje cells in vivo: temporal patterns

The output neurons of both cerebral and cerebellar cortices are known to be irregular, supported by high coefficients of variation (CV) of the interspike intervals (ISIs) measured in spike trains lasting hundreds of seconds (Softky and Koch, 1993; Vos et al., 1999; Goossens et al., 2001). However, considering the highly regular firing of simple spikes in vitro (Smith et al., 2003, Raman et al, 1999), it is not fully understood how inputs shift from regular to highly irregular firing..

Purkinje cells (PCs) are the sole output neurons of the cerebellar cortex. They generate both simple spikes (SSs) and complex spikes (CSs), but only SS are the result of the computations performed by cerebellar cortex (Ito, 1984). The cerebellum plays an important role in motor control and more particular in its dynamics by adapting the relative timing of muscle activations (Ito, 1984). It is also involved in several other tasks requiring the precise representation of temporal information (Ivry and Spencer, 2004).

Therefore it is surprising that studies of coding by PCs have usually considered only SS (Shidara et al., 1993; Coltz et al., 1999; Kahlon and Lisberger, 2000) and CS (Kitazawa et al., 1998; Goossens et al., 2004) mean firing frequencies. Little attention has been paid to the temporal structure of the spike train though spike timing has been shown to encode additional

information in many systems (Rieke et al., 1997; Vanrullen et al., 2005).

We investigated the temporal structure of spontaneous SS trains in data from two sources. Recordings from the cerebellar hemisphere of anaesthetized rats (n = 48) were compared to data from floccular PCs in awake mice (n = 37). Firing rates were similar for both data sets (45.5 ± 28.5 Hz and 51.0 ± 16.4 Hz respectively, $p > 0.2$). The CV of these spike trains was very high, 3.93 (mean, range 0.56 - 20.5) and 1.39 (0.28 - 9.76; $p < 10^{-4}$) for rat and mouse PCs respectively, comparable to previously reported values (Vos et al., 1999; Goossens et al., 2001) and suggesting very irregular firing.

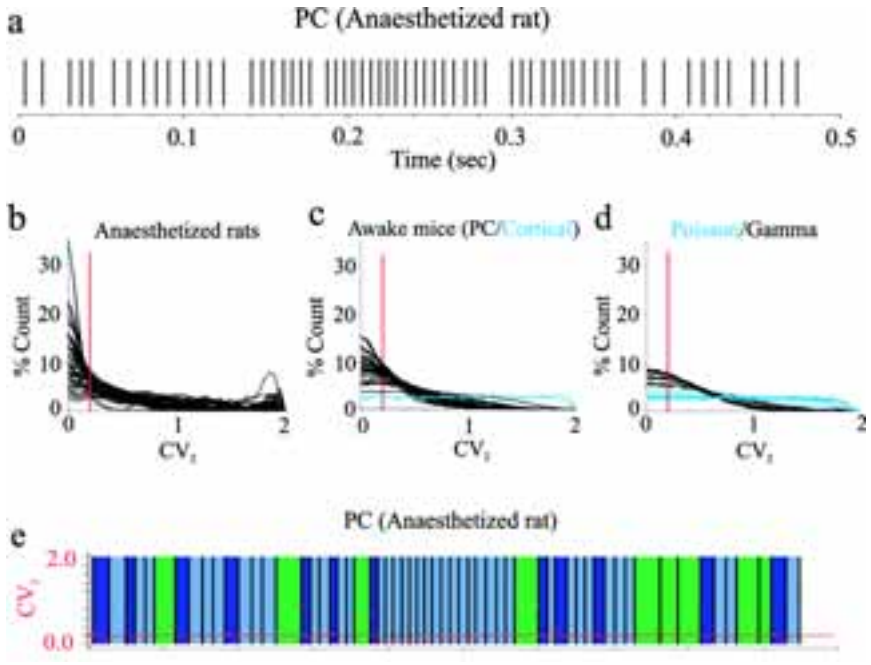

Figure 1: Regular pattern in simple spike trains.

Nevertheless, careful inspection of the spike trains showed clear patterns of regular firing (Fig 1a). We characterized these patterns by computing the CV_2, a measure of short range variability calculated over two consecutive ISIs (Holt et al., 1996). CV_2 showed more regular firing in mice, 0.39 (mean, range 0.25 - 0.73), than in rats, 0.51 (0.13 - 1.07; $p < 0.001$). In both data sets the CV_2 distribution was skewed with a high proportion of low CV_2 values (Fig 1b,c), suggesting regular spiking patterns. This was very different from CV_2 distributions in other neurons. For example, spontaneous spiking of cortical neurons showed a flat CV_2 distribution (Holt et al., 1996), similar to the realization of a homogeneous Poisson process (Fig 1c,d blue lines).

We used a threshold on the measured CV_2 values to isolate the regular spiking patterns. The procedure is illustrated in Fig. 1e: CV_2 was computed for the 2 intervals surrounding each spike. Whenever the CV_2 value was smaller or equal to 0.2 these 2 ISIs were considered part of a regular pattern (blue). If the next ISI also had a CV_2 value lower than 0.2 the following interval belonged to the same pattern, if not the ISI was either single (i.e. not belonging to any

pattern, green) or the start of a new pattern (if the following CV_2 value was lower than 0.2). Using this procedure 52% of ISIs in both data sets were classified as belonging to a pattern.

Patterns were characterized by two parameters: pattern mean ISI and pattern size, which is the total number of ISIs in the pattern. For small pattern sizes a wide range of mean ISIs was present but long patterns occurred only for short ISIs, though not for the shortest. Because small patterns are more frequent the distribution of pattern mean ISIs captured most of the peak of the overall ISI distribution and little of its tail. While these properties were similar for the two data sets, the maximum pattern sizes differed significantly. Most patterns were short, but 19.9 ± 2.9% (rat) and 11.8 ± 1.5% (mice) contained 5 or more ISIs with a maximum of up to 182 and 21 respectively.

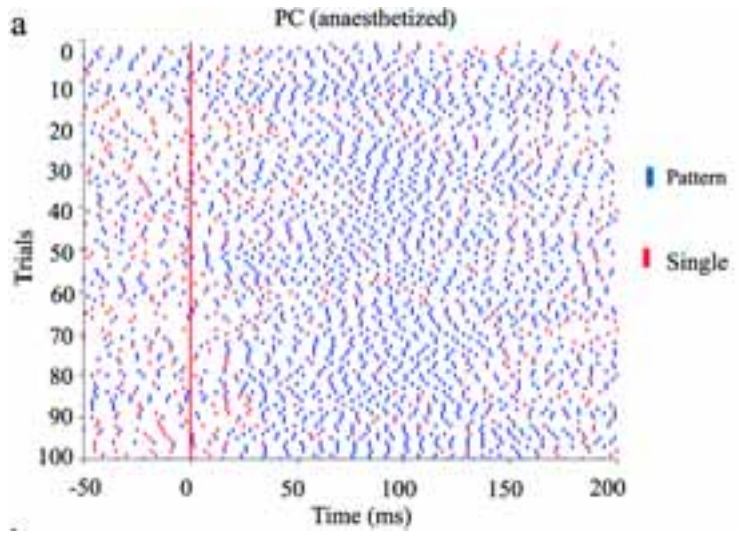

Figure 2: Stimulation changes properties of patterns.

In conclusion, we found that the CV_2 can be a measure to isolate regular firing patterns containing 2 to tens of ISIs from spontaneous SS trains in PCs. These patterns contained usually short ISIs, especially if they were long. Possibly the differences between rat and mice data can be explained by the use of anaesthesia in the former. If one assumes that the patterns form the code transmitted by PCs then one predicts faster changes in this code, i.e. shorter pattern sizes, in awake, active animals than in anaesthetized ones.

Additional support for the functional importance of the regular patterns comes from an example of rat stimulation data shown in Fig. 2. In the 200 ms following stimulation the proportion of ISIs belonging to regular patterns increased from 54.6% to 78.0%. The expected increase in PC firing frequency following stimulation was expressed as longer regular patterns with faster mean ISIs.

Our results suggest that (1) PCs may code information in their SS trains as the length and/or mean ISI of regular patterns, (2) the irregularity measured by the mean CV is caused by mixing of different regular patterns over time and (3) the regular patterns can be useful in single trial based analysis.

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

1.Interactions between reelin and its receptors

In 2002 and 2003, 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. However, there is indication that binding to lipoprotein receptors and Dab1 phosphorylation may not be sufficient to trigger the complete reelin signaling. These data were incorporated in the PhD thesis of Y. Jossin, presented in 2003, and have been published (Jossin et al., 2004).

2. Dissection of Reelin signalling in vitro

We have developed a simple slice culture for studies of cortical neuronal migration. Slices are cut at E13 and allowed to develop in culture for 2 days in defined medium and in the presence of 95% oxygen. Using this system, we could demonstrate rescue of the reeler phenotype by reelin and the central fragment of reelin that binds to receptors and phosphorylates Dab1 (see above). Using small, cell permeant inhibitors of canonical signalling pathways, we would demonstrate a key role for Src family kinases in Dab1 phosphorylation and reelin signalling. We also showed that protein kinases C are essential for neuronal cortical migration (Jossin et al, 2003). Progress on reelin signalling has been reviewed by Y. Jossin in a paper in press (Jossin, 2005).

3. New antibodies against VLDLR, ApoER2 and reelin.

We generated panels of monoclonal antibodies against the ectodomains of VLDLR and ApoER2. These Abs yield good results in western blot and immunoprecipitation and some seem to reveal a specific signal in immunohistochemistry. However, immunohistochemical results remain suboptimal despite much trials. Among the antibodies generated, two (one against ApoER2 and one against VLDLR) are able to stimulate Dab1 phosphorylation when used in combination. When these antibodies are added to reeler slices in vitro, they are unable to rescue the reeler phenotype of the slice (Jossin et al., 2004). Yet, phenotype rescue is observed with recombinant Reelin proteins that are larger than antibodies. This suggests that another event parallel to the Dab1 phosphorylation pathway may be required to fulfil the reelin signal.

As the central fragment of reelin is physiologically active, it is important to obtain antibodies directed against this part of the protein. After several unsuccessful attempts over many years, we succeeded in generating a new monoclonal antibody that recognizes this fragment. This antibody allowed us to demonstrate unequivocally the presence of all reelin fragments that result from processing, in the cerebrospinal fluid and in tissue. Furthermore, this antibody

blocks reelin signalling in biochemical experiments and perturbs migration in the slice culture assay, further indicating that the central part of the protein is indeed functionally necessary. We are presently mapping the epitope of that antibody before submitting this work for publication.

4. Reelin in neurological diseases

In collaboration with the Neurology Service from UCL/St Luc (Prof. C. Sindic), we were able to detect reelin and its processing fragments in the human CSF. We could not find any correlation between reelin immunoreactivity and age or neurological disease. In three patients with chronic schizophrenia, no evident modifications of reelin levels were detected. These results have been published (Ignatova et al., 2004). Unfortunately, for personal reasons, Ms Ignatova decided not to pursue her PhD further and left the laboratory end 2003.

5. Reelin and cortical evolution

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 several amniote lineages in order to assess this idea further. Crododilians were still lacking in our analysis because we could not obtain embryos. With help from the "Ferme aux Crocodiles" (Pierrelatte, france), we carried out a study of reelin expression in embryonic crocodiles (Tissir et al., 2003). A key point in our comparative studies is that all amniotes have horizontal, early generated reelin positive neurons in their cortical marginal zone, suggesting that these cells derive evolutionarily from a primitive type present in stem amniotes or before, and that mammalian Cajal-Retzius types are a highly specialized version of this primitive cell. In mammals, Cajal-Retzius neurons are characterized by co-expression of reelin and the oncogène p73. Co-expression of reelin and p73 in horizontal cells in other amniotes would thus provide a strong argument in favour of the hypothesis formulated above. In order to test this hypothesis, we cloned by RT-PCR parts of the reelin and p73 cDNA in chick, turtle, crocodile, lizard and mouse and carried out double in situ hybridization studies in embryonic cortices using species-specific DIG labelled reelin and radioactive p73 probes. This work showed colocalisation of reelin and p73 transcripts in neurons in the embryonic marginal zone in mice, turtle and lizard, suggesting that these cells are indeed descendants from a same cell. Unfortunately, the colocalization is much less clear in lizards and chick, that have very few p73 positive neurons in their embryonic cortex, only a minority of which express reelin. A likely explanation for this lays in the action of p73, which is probably implicated in the control of apoptosis of CR cells, a control that is perhaps not required to the same extent in all species. Unfortunately, this situation does not provide much argument for or against our working hypothesis, investigation of which awaits the identification of new markers. We therefore decided not to pursue this work further. Our results and the working hypothesis will be published as a chapter in a large volume on Brain Evolution under preparation by Elsevier (Tissir and Goffinet, 2005).

6. The Dab1 gene

In collaboration with I. Bar and C. Lambert, we completed and published an extensive study of the Dab1 gene in mouse and man (Bar et al., 2003). The complexity of this gene and its

transcriptional regulation is really mind-boggling. We do not see how we can make significant progress in the understanding of Dab1 expression and this project has not been pursued further.

7. Potential effectors of the reelin pathway

The results of several Representational Difference Analysis (RDA) and Differential Display (DD) experiments have been analyzed. A technical improvement of the RDA procedure has been published (Kuvbachieva et al., 2004). Four novel genes bear some interest, particularly one of them, named clone 61, is expressed in the embryonic cortical plate and developmentally regulated. The genomic structure has been defined. Northern blots and RT-PCR studies have been done, and EGFP fusion proteins produced in transfected HEK293 cells for studies of intracellular distribution. This work, carried out in collaboration with the laboratory of M. Simonneau (INSERM, Paris) has been published (Kuvbachieva et al., 2004). Pursuing this project further would require to produce a mouse knock-out for gene 61. Producing a KO mouse is a huge effort for our small team. Furthermore, our priority goes to the Celsr3 project explained below. Therefore, studies of gene 61 will not be pursued further in the near future.

8. Screening of small molecules for interference with brain cell migration

Following on the development of our slice culture system (Jossin, 2003), we decided to screen a bank of chemical compounds. This screen aims at identifying molecules that have preferential toxicity for migrating brain cells and are thus candidates for development of anticancer compounds, and for molecules that perturb migration and cortical formation and are candidate inhibitors of the reelin signal or other signals required for migration. Following selection of our project by the National Cancer Institute, we obtained the bank of 1990 leading compounds (Diversity Set) and have performed a first pass by screening pools of 5-8 molecules at a target concentration of 10 µM. Thus far, we have identified and verified the action of 10 molecules that perturb migration and of 70 molecules that show toxicity for migrating cells. Among the latter, our screen identified more than 10 molecules already known and investigated as potential drugs of interest in cancer research (such as a ellipticine, bouvardine, topotecan), providing proof of principle that our screening procedure identifies indeed valuable candidates. A first manuscript describing this approach is under preparation.

9. The protocadherin Celsr3

In 2001, we decided to invest some effort in studies of Celsr protocadherins, proteins that are the orthologs of starry night/flamingo (stan/fmi) in Drosophila. In flies, mutations in these genes affect epithelial polar cell polarity, dendritic and axonal deployment, and the different functions of the fly protein are probably carried out by the three orthologs Celsr1-3 in mammals. We begun by comparing the expression patterns of Celsr1, 2 and 3, during embryonic development. This analysis was very useful, as it showed that all three genes are specifically expressed in the central nervous system with overlapping, yet largely complementary patterns (Tissir et al., 2002). Celsr3 was selected for further study because it is expressed mostly in maturing neurons and is downregulated during development. It was thus a candidate for interaction with reelin signalling. Based on work in Drosphila that suggested interaction between stan/fmi and a new gene named Diego, we cloned the mouse ortholog of the Diego gene, Alkrd6, which encodes a protein with ankyrin repeats. However, the expression pattern

did not correlate with any Celsr canvas and we did not study that gene further (Tissir et al., 2002). In collaboration with the Neurobiology Laboratory in Namur (O. De Backer, I. Bar and C. Lambert de Rouvroit), we decided to inactivate Celsr3 by homologous recombination is ES cells and to produce knock-out mice. These mice became available in the spring 2004. The phenotype does not suggest any interaction with reelin signalling as we thought, but is highly interesting. The mice die at birth of respiratory failure of central origin. They have a very important brain malformation, with absence of anterior commissure, of internal capsule, of the medial lemniscus and several other longitudinal bundles. Intriguingly, this phenotype is identical to the malformation in mice with inactivation of Frizzled-3 (generated in the laboratory of J. Nathans, Johns Hopkins Univ., Baltimore). Thus, Celsr3 and Fzd3 define a new genetic pathway that plays a critical role in the development of the early connectivity. We believe that this observation is extremely important and have decided that investigating the role of Celsr3 in brain development will be a priority of our laboratory in the coming years. A first manuscript is under review.

10. Reviews on Reelin and cortical development.

Two reviews (submitted to referees), one focused on reeler mice and reelin signalling (Tissir and Goffinet, 2003), and the other with a wider perspective on early cortical development (Jossin, 2005) have been published.

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Glio-vascular calcium signaling and neurovascular metabolic coupling in the brain.

Neuronal electrochemical impulses are traditionally considered as the basis of information processing in the brain. There is, however, growing evidence that 'non-excitable' cell types such as glial cells and also brain vessel cells, are actively participating in brain functioning by responding to synaptic activity, by modulating these information circuits and by exchanging signals to coordinate the functioning of the basic triad consisting of neurons, glial cells and microvascular cells that together constitute the neurovascular unit. Intracellular calcium ions play a pivotal role in non-excitable cells as a messenger for intracellular and intercellular signaling. Because there is a certain degree of homology between action potentials and calcium transients these signals provide non-excitable cells with a form of excitability that is called 'calcium excitability'. Intercellular calcium signals are transient changes in cytoplasmic free calcium that are, analogously to action potentials in neurons, characterized by an initiating trigger followed by a mechanism that propagates the calcium signal to neighboring cells. The spectrum of intercellular calcium signals ranges from the most elemental form of calcium signal exchange between just a pair of cells up to massive intercellular calcium waves encompassing hundreds of cells. The communication of calcium signals between brain cells is a typical feature of glial cells, but it is not restricted to these cells and also includes neurons and microvascular cells. We have investigated the mechanisms and role of communicated calcium signals between glial cells and microvascular cells[1].

Astrocytes are intermediately positioned between neurons and microvascular cells and therefore occupy a key signaling position between these two important players. Astrocytes are in contact with smooth muscle cells of arterioles, which determine the vessel diameter and thus blood flow, and with endothelial cells of capillary vessels, which form the blood-brain barrier where important transports take place. Neuronal activity can trigger calcium signals in astrocytes and work of our group has demonstrated in a co-culture model that astrocytes can communicate these calcium signals further towards the capillary endothelial cells. We identified two mechanisms that, at least *in vitro*, support bidirectional astrocyte-endothelial and also endothelial-endothelial calcium signal communication: the first mechanism involves the diffusion of the calcium mobilizing messenger $InsP_3$ through gap junction channels and the second relies on paracrine signaling involving the release of ATP, diffusion in the extracellular space, binding to receptors on neighboring cells and activation of downstream signaling cascades that lead to an increase of cytoplasmic free calcium in the target cell[2]. We further investigated the mechanism of endothelial ATP release and found that this is in large part mediated by connexin-related mechanisms[3,4]. Work with peptides that mimic a short sequence of the connexin-43 subunit revealed drastic inhibitory effects on cellular ATP release, supporting the hypothesis that the release pathway is formed by connexin hemichannels. Connexin hemichannels are half gap junction channels that, in contrast to gap junction channels, are not involved in cell coupling but form a large conductance conduit between the cells' interior and the extracellular space[5]. Further work is directed towards the role of

intracellular calcium as a trigger to activate this new release pathway, to determine its involvement relative to the vesicular release pathway and to investigate its regulation by various kinases. Recent results have demonstrated that an increase of intracellular calcium can open connexin-32 hemichannels to release ATP or allow the influx of extracellular molecules into the cells in a calmodulin-dependent manner[6]. Various kinases furthermore affect connexin hemichannels and gap junction channels in a different manner, suggesting that hemichannels are differently regulated as compared to gap junction channels[7].

ATP release by astrocytes and endothelial cells is not only involved in astrocyte-endothelium calcium signal communication but is also an essential element of the paracrine communication pathway between the blood vessels and the blood cells. Endothelial ATP has indeed been demonstrated to act as a proinflammatory signal on blood immune cells such as leukocytes and lymphocytes. We have put forward the hypothesis that ATP release through connexin hemichannels forms a high-capacity mechanism that may act to overcome dilution and washout of the endothelial ATP signal by the blood flow, based on the fact that a single stimulus with $InsP_3$ triggers the release of quite a substantial fraction (1-2 %) of the cellular ATP pool[8]. Interactions of immune cells with capillary endothelial cells are also important in the disruption of the blood-brain barrier associated with neuroinflammatory diseases such as mutliple sclerosis and AIDS-HIV dementia. The opening of the barrier involves the action of cytokines like TNF-α, IL1-β and IFN-γ and an increase of endothelial cytoplasmic calcium. Our working hypothesis is that calcium signals communicated between endothelial cells may act to spatially spread and thus amplify the calcium-induced opening of the blood-brain barrier. We investigated whether TNF-α has a modulatory influence on the communication of calcium signals between capillary endothelial cells. We found that this cytokine inhibits two connexin-related communication pathways namely gap junction channels and connexin hemichannels. TNF-α appeared to block ATP release through connexin hemichannels and as a consequence, all types of purinergic signaling -not only purinergic calcium signaling- are predicted to be silenced by this cytokine[2]. Silencing of purinergic signaling at the blood-brain barrier is likely to profoundly influence the complex interactions of blood immune cells with blood-brain barrier endothelial cells.

Several possibilities should be considered concerning the role of astrocyte-endothelial calcium signal communication[9]. Changes of endothelial calcium are considered a key step in disrupting the tight junctions between endothelial cells, thereby opening the blood-brain barrier, and astrocyte-endothelial calcium signals might thus be involved in the process of barrier opening under pathological conditions. Under physiological conditions, endothelial calcium signals may communicate a vasodilating message to the vascular smooth muscle cells; alternatively, endothelial calcium signals may influence the transports occurring over the blood-brain barrier. We propose that astrocyte-endothelial calcium signals play a role in 'neurobarrier coupling', that is coupling of neuronal activity to the transports over the blood-brain barrier[10]. Neurobarrier coupling, together with neurovascular and neurometabolic coupling, contributes

to adapting the transport of glucose over the barrier to the local astrocytic and neuronal needs[10]. Preliminary work suggests that certain neurotransmitters acting on endothelial calcium are indeed able to stimulate glucose uptake in endothelial cells and we are preparing to set up an in vitro blood-brain barrier model in order to investigate this question in full detail.

In recent work, we have investigated calcium signal communication in brain slices. Brain slices were acutely isolated from mouse brain (P10-12) and were cut along a coronal plane to visualize the pial vessels penetrating the cortical layers. Electrical stimulation of the neural tissue approximately 100 µm away from a small vessel (±12 µm in diameter), triggered intercellular calcium waves in the glial cells that were communicated towards endothelial cells and smooth muscle cells of the vessel (unpublished observation). In accordance to these observations, the vessels reacted with either dilation or constriction, which is likely to be explained by communication of the calcium signal either to the endothelial cells, resulting in the communication of a secondary vasodilating message to the smooth muscle cells, or directly to the smooth muscle cells causing them to contract. Further work will be directed to elucidate the complex vessel responses to neuronal electrical stimulation, but these experiments already illustrate that glial-vascular calcium signals can be observed in a preparation resembling the complex organization of the in vivo brain.

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Part I : Alzheimer's disease : cellular and molecular aspects.

1.1. Introduction.

Alzheimer's disease (AD) is a neurodegenerative disease leading to dementia. The frequency of the disease increases with age. At the age of 85, 25 to 30 % of the population is affected. Since high sensitive and specific biological markers are not yet available, the clinical diagnosis of the disease has to be confirmed by post-mortem neuropathological examination of patients brains. The diagnosis of AD is confirmed by the co-existence of two types of histological lesions in the brain: intracellular neurofibrillary tangles and extracellular senile plaques. The neurofibrillary tangles are made of Paired Helicoïdal Filaments (PHF) containing the microtubule-associated protein Tau. In AD, Tau is hyperphosphorylated, dissociates from microtubules and aggregates into PHF. The extracellular senile plaques contain an amyloid core, the major constituent of which being a small 39 to 43 aminoacids peptide called β-amyloid peptide or Aβ. Aβ is produced from a larger precursor, the Amyloid Precursor Protein (APP). The expression of human APP in several cell lines allowed to identify two catabolic pathways of the protein:

- The non-amyloidogenic pathway in which APP is cleaved by an α-secretase activity. The α-cleavage of APP occurs within the Aβ sequence, precluding its formation. Candidate α-secretase activities, ADAM 10 and TACE proteins, belong to the disintegrin/metalloprotease family.
- The amyloidogenic pathway in which APP is sequentially cleaved by β- and γ-secretase activities to release Aβ. The β-secretase has been identified as the BACE 1 protein, whereas the γ-secretase activity is not fully characterized. However, it is commonly admitted that the γ-secretase activity is part of a multiproteic complex containing at least the Presenilin 1 (PS1), Nicastrin, Pen-2 and Aph-1 proteins.

Thanks to the Queen Elisabeth Medical Foundation, we have studied the metabolism of human APP in rat cultured neurons. A sustained increase of cytosolic calcium induces the production of intraneuronal Aβ leading to important neuronal toxicity. In addition, we have studied the role of PS1 in the γ-cleavage of APP. PS1 stabilizes the substrate of the γ-secretase actvity but is not able to cleave APP.

1.2. Correlation between beta-amyloid peptide production and human APP-induced neuronal death.

Kienlen-Campard P, Octave JN. Peptides. 2002 Jul;23(7):1199-204.

The production of amyloid peptide (Aβ) from its precursor (APP) plays a key role in Alzheimer's disease (AD). However, the link between Aβ production and neuronal death remains elusive. We studied the biological effects associated with human APP expression and metabolism in rat cortical neurons. Human APP expressed in neurons is processed to produce Aβ and soluble APP. Moreover, human APP expression triggers neuronal death. Pepstatin A, an inhibitor of aspartyl proteases that reduces Aβ production, protects neurons from APP-induced neurotoxicity. This suggests that Aβ production is likely to be the critical event in the neurodegenerative process of AD.

1.3. Intracellular amyloid-beta 1-42, but not extracellular soluble amyloid-beta peptides, induces neuronal apoptosis.

Kienlen-Campard P, Miolet S, Tasiaux B, Octave JN. J Biol Chem. 2002 May 3;277(18):15666-70.

Alzheimer disease (AD), the most frequent cause of dementia, is characterized by an important neuronal loss. A typical histological hallmark of AD is the extracellular deposition of beta-amyloid peptide (Aβ), which is produced by the cleavage of the amyloid precursor protein (APP). Most of the gene mutations that segregate with the inherited forms of AD result in increasing the ratio of Aβ 42/Aβ 40 production. Aβ 42 also accumulates in neurons of AD patients. Altogether, these data strongly suggest that the neuronal production of Aβ 42 is a critical event in AD, but the intraneuronal Aβ 42 toxicity has never been demonstrated. Here, we report that the long term expression of human APP in rat cortical neurons induces apoptosis. Although APP processing leads to production of extracellular Aβ 1-40 and soluble APP, these extracellular derivatives do not induce neuronal death. On the contrary, neurons undergo apoptosis as soon as they accumulate intracellular Aβ 1-42 following the expression of full-length APP or a C-terminal deleted APP isoform. The inhibition of intraneuronal Aβ 1-42 production by a functional gamma-secretase inhibitor increases neuronal survival. Therefore, the accumulation of intraneuronal Aβ 1-42 is the key event in the neurodegenerative process that we observed.

1.4. Intraneuronal amyloid-beta1-42 production triggered by sustained increase of cytosolic calcium concentration induces neuronal death.

Pierrot N, Ghisdal P, Caumont AS, Octave JN. J Neurochem. 2004 Mar;88(5):1140-50.

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the presence in the brain of senile plaques which contain an amyloid core made of beta-amyloid peptide (Abeta). Abeta is produced by the cleavage of the amyloid precursor protein (APP). Since impairment of neuronal calcium signalling has been causally implicated in ageing and AD, we have investigated the influence of an influx of extracellular calcium on the metabolism of human APP in rat cortical neurones. We report that a high cytosolic calcium concentration, induced by neuronal depolarization, inhibits the alpha-secretase cleavage of APP and triggers the accumulation of intraneuronal C-terminal fragments produced by the beta-cleavage of the protein (CTFbeta). Increase in cytosolic calcium concentration specifically induces the production of large amounts of intraneuronal Abeta1-42, which is inhibited by nimodipine, a specific antagonist of l-type calcium channels. Moreover, calcium release from endoplasmic reticulum is not sufficient to induce the production of intraneuronal Abeta, which requires influx of extracellular calcium mediated by the capacitative calcium entry mechanism. Therefore, a sustained high concentration of cytosolic calcium is needed to induce the production of intraneuronal Abeta1-42 from human APP. Our results show that this accumulation of intraneuronal Abeta1-42 induces neuronal death, which is prevented by a functional gamma-secretase inhibitor.

1.5. Failure of the interaction between presenilin 1 and the substrate of gamma-secretase to produce Abeta in insect cells.

Pitsi D, Kienlen-Campard P, Octave JN.J Neurochem. 2002 Oct;83(2):390-9.

Aggregates of beta-amyloid peptide (Abeta) are the major component of the amyloid core of the senile plaques observed in Alzheimer's disease (AD). Abeta results from the amyloidogenic processing of its precursor, the amyloid precursor protein (APP), by beta- and gamma-secretase activities. If beta-secretase has recently been identified and termed BACE, the identity of gamma-secretase is still obscure. Studies with knock-out mice showed that presenilin 1 (PS1), of which mutations are known to be the first cause of inherited AD, is mandatory for the gamma-secretase activity. However, the proteolytic activity of PS1 remains a matter of debate. Here we used transfected Sf9 insect cells, a cellular model lacking endogenous beta- and/or gamma-secretase activities, to characterize the role of BACE and PS1 in the amyloidogenic processing of human APP. We show that, in Sf9 cells, BACE performs the expected beta-secretase cleavage of APP, generating C99. We also show that C99, which is a substrate of gamma-secretase, tightly binds to the human PS1. Despite this interaction, Sf9 cells still do not produce Abeta. This strongly argues against a direct proteolytic activity of PS1 in APP processing, and points toward an implication of PS1 in trafficking/presenting its substrate to the gamma-secretase.

1.6. Presenilin 1 stabilizes the C-terminal fragment of the amyloid precursor protein independently of gamma-secretase activity

Pitsi D, Octave JN. J Biol Chem. 2004 Jun 11;279(24):25333-8.

The cleavage of the transmembrane amyloid precursor protein (APP) by beta-secretase leaves the C-terminal fragment of APP, C99, anchored in the plasma membrane. C99 is subsequently processed by gamma-secretase, an unusual aspartyl protease activity largely dependent on presenilin (PS), generating the amyloid beta-peptide (Abeta) that accumulates in the brain of patients with Alzheimer's disease. It has been suggested that PS proteins are the catalytic core of this proteolytic activity, but a number of other proteins mandatory for gamma-secretase cleavage have also been discovered. The exact role of PS in the gamma-secretase activity remains a matter of debate, because cells devoid of PS still produce some forms of Abeta. Here, we used insect cells expressing C99 to demonstrate that the expression of presenilin 1 (PS1), which binds C99, not only increases the production of Abeta by these cells but also increases the intracellular levels of C99 to the same extent. Using pulse-chase experiments, we established that this results from an increased half-life of C99 in cells expressing PS1. In Chinese hamster ovary cells producing C99 from full-length human APP, similar results were observed. Finally, we show that a functional inhibitor of gamma-secretase does not alter the ability of PS1 to increase the intracellular levels of C99. This finding suggests that the binding of PS1 to C99 does not necessarily lead to its immediate cleavage by gamma-secretase, which could be a spatio-temporally regulated or an induced event, and provides biochemical evidence for the existence of a substrate-docking site on PS1.

Part 2 : Biochemical basis of the neurotoxicity in neurodegenerative diseases : focus on glutamate transporters

1.1. Introduction

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 neuronal transmission. In addition to its role in the transmission of excitation inputs, glutamate is involved in complex activities, including learning, memory and synaptic plasticity. While ionotropic receptors induce rapid responses and are involved in the transmission of glutaminergic responses, 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. 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 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, during year 2002-4, the regulation of glutamate transport (and glutamate transporter expression) has been studied in models of primary cultured astrocytes and in stem cells exposed to growth factors favouring their differentiation into glial-like cells.

1.2. Summaries of the recent studies

The first ongoing project concerns the study of the molecular and cellular mechanisms involved in the regulation of glutamate transporters in primary cultured astrocytes.

In vitro culture of astroglial progenitors can be obtained from early post natal brain tissues and several methods have been reported for promoting their maturation into differentiated

astrocytes. Hence, a combination of several nutriments/growth factors - the G5 supplement (insulin, transferrin, selenite, biotin, hydrocortisone, fibroblast growth factor and epidermal growth factor) - is widely used as a culture additive favouring the growth, differentiation and maturation of primary cultured astrocytes. Considering the key role played by glial cells in the clearance of glutamate in the synapses, cultured astrocytes are frequently used as a model for the study of glutamate transporters. Indeed, it has been shown that when tested separately, growth factors influence the expression and activity of the GLAST and GLT-1. The present study aimed at characterising the functional expression of these transporters during the time course of differentiation of cultured cortical astrocytes exposed to the supplement G5. After a few days, the vast majority of cells exposed to this supplement adopted a typical stellate morphology (fibrous or type II astrocyte) and showed intense expression of the glial fibrillary acidic protein. Both RT-PCR and immunoblotting studies revealed that the expression of both GLAST and GLT-1 rapidly increased in these cells. While this was correlated with a significant increase in specific uptake of radiolabelled aspartate, fluorescence monitoring of the Na^+ influx associated with glutamate transporters activity revealed that the exposure to the G5 supplement considerably increased the percentage of cells participating in the uptake. Biochemical and pharmacological studies revealed that this activity did not involve GLT-1 but most likely reflected an increase in GLAST-mediated uptake. Together, these data indicate that the addition of this classical combination of growth factors and nutriments drives the rapid differentiation toward an homogenous culture of fibrous astrocytes expressing functional glutamate transporters.

Endothelin-1 (ET-1) is a 21 amino acid peptide that exerts several biological activities through interaction with specific G-protein coupled receptors. Increased ET-1 expression is frequently associated with pathological situations involving alterations in glutamate levels. In the present study, a brief exposure to ET-1 was found to increase aspartate uptake in C6 glioma cells, which endogenously express the neuronal glutamate transporter EAAC1 (pEC50 of 9.89). The stimulatory effect of ET-1 mediated by ET_A receptors, corresponds to a 62% increase in the V_{max} with no modification of the affinity for the substrate. While protein kinase C activity is known to participate in the regulation of EAAC1, the effect of ET-1 on the glutamate uptake was found to be independent of this kinase activation. In contrast, inactivation of $G_{o/i}$ type G-protein dependent signaling with pertussis toxin was found to impair ET-1-mediated regulation of EAAC1. Examination of the cell surface expression of EAAC1 by protein biotinylation studies or by confocal analysis of immuno-fluorescence staining demonstrated that ET-1 stimulates EAAC1 translocation to the cell surface. Hence, disruption of the cytoskeleton with cytochalasin D prevented ET-1-simulated aspartate uptake. Together, the data presented in the current study suggest that ET-1 participate in the acute regulation of glutamate transport in glioma cells. Considering the documented role of glutamate excitotoxicity in the development of brain tumors, endothelinergic system constitutes a putative target for the pharmacological control of glutamate transmission at the vicinity of glioma cells.

The second ongoing project concerns the characterization of the expression of glutamate transporters in astrocytic cells derived from neural stem cells or from bone marrow purified mesenchymal stems cells.

The possibility to isolate stem cells from the adult central nervous system and to maintain and propagate these cells in vitro has raised a general interest with regards to their use in cell replacement therapy for degenerative brain diseases. Considering the critical role played by astrocytes in the control of glutamate homeostasis, we have characterised the expression of functional glutamate transporters in neural stem cells exposed to selected culture conditions favouring their differentiation into astrocytes. Commonly, neural stem cells proliferate in suspension as neurospheres in serum free medium. The addition of serum or a supplement of growth factors (G5) to the culture medium was found to trigger cell adhesion on coated surfaces and to favour their differentiation. Indeed, after 7 days in these conditions, the vast majority of the cells adopted markedly distinct morphologies corresponding to protoplasmic (with serum) or fibrous (with G5 supplement) astrocytes and approximately 35-40% acquired the expression of the glial fibrillary acidic protein (GFAP). Immunocytochemical analysis also revealed that the treatments with serum or with the G5 supplement triggered the expression of the glial glutamate transporters GLT-1 (35 and 21%, respectively) and GLAST (29 and 69 %, respectively). This effect was correlated with a robust increase in the Na^+-dependent [^{3}H]-D-aspartate uptake, which was partially inhibited by dihydrokainate, a selective blocker of GLT-1. Together, these results indicate that in vitro differentiation of cultured neural stem cells can give rise to distinct populations of astrocytes expressing functional glutamate transporters.

Adult bone marrow mesenchymal stem cells are multipotent cells that can differentiate into a variety of mesodermal tissues. Recent studies have reported on their ability to also evolve into non-mesodermal cells, especially into neural cells. While most of these studies revealed that manipulating these cells triggers the expression of typical nervous markers, less is known about the induction of neuronal- or glial-related physiological properties. The present study was focused on the characterisation of glutamate transporters expression and activity in rat mesenchymal stem cells grown in culture conditions favouring their differentiation into astroglial cells. Ten days exposure of the cells to the culture supplement G5 was found to increase the expression of nestin (neuro-epithelial stem cell intermediate filament), an intermediate filament protein expressed by neural stem cells. Simultaneously, a robust induction of the high-affinity glutamate transporter GLT-1 (and GLAST) expression was detected by RT-PCR and immunocytochemistry. This expression was correlated with a highly significant increase in the Na^+-dependent [^{3}H]-D-aspartate uptake. Finally, while GFAP immunoreactivity could not be detected, the induced expression of the astrocytic enzyme glutamine synthetase was demonstrated. These results indicate that in vitro differentiation of adult mesenchymal stem cells in neural precursors coincides with the induction of functional glutamate transport systems. Although the astrocytic nature of these cells remains to be confirmed, this observation gives support to the study of mesenchymal stem cells as a promising tool for the treatment of neurological diseases involving glutamate excitoxicity.

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1. Introduction

The present activity report reflects the research efforts made in 3 main directions : (i) the characterization of sleep/wakefulness regulation; (ii) the assessment of persisting brain responses in unconscious patients and (iii) the study of controlled and automatic processes.

As it appears clearly, the activity of our group gracefully combines multiple neuroimaging techniques, including PET, fMRI, and EEG.

Present research further combines these and other techniques. A special effort is being made to record high quality multichannel EEG signal during continuous fMRI acquisitions.

2. Regulation of sleep and wakefulness

2.1. Study of the human non image forming visual system

The brain processes light information to visually represent the environment but also to detect changes in ambient light level. The latter information induces non-image forming responses mediated by neural systems partially distinct from the visual system, and exerts powerful effects on physiology such as synchronization of the circadian clock. In rodents, irradiance information is transduced from a discrete subset of photosensitive retinal ganglion cells via the retinohypothalamic tract to various hypothalamic regulatory structures including the hypothalamic suprachiasmatic nuclei, the master circadian pacemaker. In humans, light acutely modulates alertness but the cerebral correlates of this effect are unknown. In a first positron emission tomography study, we showed that during the biological night, light exposures of different durations modulate the regional cerebral blood flow of subjects attending to auditory and visual stimuli in darkness. Functional data revealed that activity in a large-scale occipito-parietal attention network and in the hypothalamus was related to the duration of light exposures. These results are now published :

Cerebral correlates of non-image forming responses following bright light exposure in Humans. Fabien Perrin, Philippe Peigneux, Sonia Fuchs, Stéphane Verhaeghe, Steven Laureys, Benita Middleton, Christian Degueldre, Guy Del Fiore, Gilles Vandewalle, Evelyne Balteau, Robert Poirrier, Vincent Moreau, André Luxen, Pierre Maquet1, Derk-Jan Dijk Current Biology, 2004, 14, 1842-6.

During 2004, we extended these results and observed the neural correlates of such a non-image forming (NIF) response to light during the daytime (i.e. biological day) using functional magnetic resonance imaging (fMRI).

Thirteen healthy subjects (7 females; age: 22 years +/-1.82; BMI: 22.2+/-2.4) followed a 7-day constant sleep schedule before being recorded twice, 2-days apart. On both visits, they stayed in dim light for 3 hours (<5lux) before performing six 8-minute auditory oddball sessions in a 3T Allegra MR scan. They had to count the number of odd sounds and respond to them by pressing a button. All sessions were performed in darkness (<0.01lux) except for session 3 and 4 of one visit where volunteers had one eye exposed to a bright white polychromatic light (>7000lux). Light was randomly given during the first or the second visit in a balanced manner over subjects.

Data were analysed using SPM2 (http://www.fil.ion.ucl.ac.uk/spm/spm2). The statistical analysis looked for a visit (light vs darkness) x sessions (5vs2) interaction, i.e. the changes in brain response related to the light exposure and not accounted for by simple repetition of sessions. The individual summary statistics images were used in a 2nd level, i.e. a random effect analysis. Inference ($p<0.05$) was corrected for multiple comparison on small volumes of interest.

A significant interaction was observed in the right superior temporal gyrus [44 -16 -8; Z-value = 4.18; p_{svc} = 0.025], the left cingulate gyrus [-20 -40 54; Z-value = 3.81; p_{svc} = 0.033], the superior frontal sulcus [18 6 74; Z-value = 3.53; p_{svc} = 0.026], the frontopolar cortex [-24 56 -2; Z-value = 3.33; p_{svc} = 0.042], and in the right intraparietal sulcus (rIPS) [24 -56 38; Z-value = 3.62; p_{svc} = 0.046, see figure 1]. As shown by the beta estimates (figure 1), the progressive decrease in activity observed in these areas when sessions are repeated in darkness is counteracted by light exposure.

In conlucion, we report for the first time neural correlates of a daytime NIF response to bright light using an oddball paradigm in fMRI, taking into account a potential order effect.

The publication summarizing these results is in preparation.

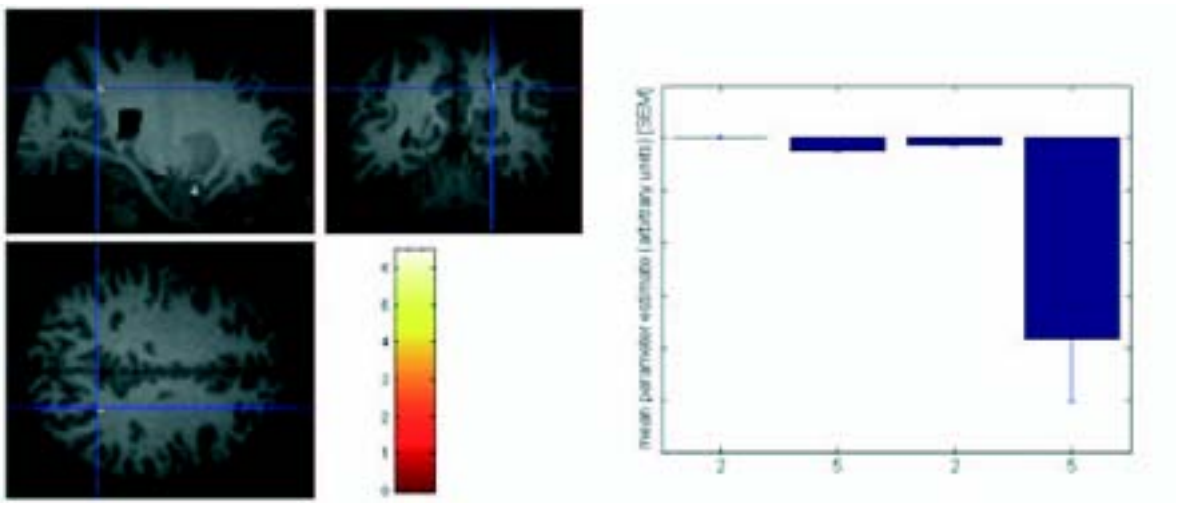

Figure 1: Significant visit by session interaction in the rIPS, displayed over the normalized structural scan of a representative subject at $p<0.001$ uncorrected. Below: beta estimates in the rIPS across sessions ($2\ 5_{light}$ / $2\ 5_{darkness}$).

2.2. Neural correlates of sigma and delta activity during human sleep

We aimed at characterizing the neural correlates of delta activity during Non Rapid Eye Movement (NREM) sleep in non sleep deprived normal young adults, based on the statistical analysis of a large positron emission tomography (PET) sleep data set. One-hundred fifteen PET scans were obtained using $H_2{}^{15}O$ under continuous polygraphic monitoring during stages 2-4 of NREM sleep. Covariations between regional cerebral blood flow (rCBF) and delta power (1.5-4 Hz) spectral density were analysed using statistical parametric mapping (SPM2). Significant negative correlations of rCBF with delta power during NREM sleep were located in the ventromedial prefrontal cortex, the basal forebrain, the striatum, the anterior insula and

the precuneus. These regions embrace the set of brain areas in which rCBF decreases during SWS as compared to REM sleep and wakefulness (Maquet et al., J Neurosci 1997), supporting the notion that delta activity is a valuable prominent feature of NREM sleep. A strong association between delta power and rCBF was observed in the ventromedial prefrontal regions, in agreement with electrophysiological studies. In contrast to the results of a previous PET study investigating the brain correlates of delta activity (Hofle et al., J Neurosci 1997), in which waking scans were mixed with NREM sleep scans, no correlation was found in the thalamus. This latter result stresses the importance of an extra-thalamic delta rhythm among the synchronous NREM sleep oscillations. Consequently, this rCBF distribution might preferentially reflect the cellular processes involved in the generation of cortical delta waves during NREM sleep.

The study has been reported in international conferences and the manuscript gathering these results is provisionally accepted for publication in NeuroImage.

2.3. Neural correlates of hear rate variability during human REM sleep

Rapid eye movement sleep (REMS) is associated with an instability in autonomous (in particular in cardiovascular) regulation. The neural mechanisms underpinning the variability of heart rate (VHR) during REMS are not known in detail, especially in humans, although during wakefulness the right insula is implicated in cardio-vascular regulation.

The analysis is based on 95 scans (54 scans in REMS and 41 during wakefulness) collected in 19 subjects in the framework of several positron emission tomography (PET) studies of human sleep, during which cerebral blood flow (CBF) was iteratively measured using the $H_2{}^{15}O$ infusion method. Complete polysomnographic recordings were obtained during the scanning sessions. Data analysis (SPM99) looked for the main effects of conditions (wake or REMS), VHR and for the condition by VHR interaction. Functional connectivity was characterized by psycho-physiological interactions ('physiological' factor : CBF in the amygdala; 'psychological' factor : REMS versus wakefulness).

The results show that in the right amygdaloïd complex, the regional CBF is related to VHR more tightly during REMS than during wakefulness (significant condition x VHR interaction, $p < 0.05$ SVC corrected). Psychophysiological interactions show that the activity in the right insula was positively related to the amygdala during wakefulness but not during REMS.

These findings suggest that the amygdala actively participates in the regulation of heart rate during REMS. The functional connectivity between the amygdala and the insular cortex, two brain areas involved in cardio-vascular regulation, differs significantly in REMS as compared to wakefulness.

The study has been reported in international conferences and the manuscript gathering these results is submitted.

2.4. Learning-dependent reactivations of brain activity during human sleep

In rats, the firing sequences observed in hippocampal ensembles during spatial learning are replayed during subsequent sleep, suggesting a role for post-training sleep periods in the off-line processing of spatial memories. Using positron emission tomogrpahy and regional cerebral blood flow measurements, we showed that in humans, hippocampal areas activated during route learning in a virtual town are likewise activated during subsequent slow wave sleep. Most importantly, we found that the amount of hippocampal activity expressed during slow wave sleep positively correlates with the improvement of performance in route retrieval on the next day. These findings suggest that learning-dependent modulation in hippocampal activity during human sleep reflects the offline processing of recent episodic and spatial memory traces, which eventually leads to the plastic changes underlying the subsequent improvement in performance. The manuscript gathering these results is published :

Peigneux, P. Laureys, S. Fuchs, S. Collette, F. Perrin, F. Reggers, J. Phillips, C. Degueldre, C. Del Fiore, G., Aerts, J. Luxen, A. Maquet, P. Are Spatial Memories Strengthened in the Human Hippocampus during Slow Wave Sleep? Neuron, 44 (2004): 535-45

2.5. Off-line persistence and early reorganization of neural activity in learning-dependent networks

Using functional neuroimaging techniques, we have previously showed learning-dependent modulations in regional cerebral activity during post-learning sleep in humans (Maquet et al., 2000; Peigneux et al., 2004; Peigneux et al., 2003). The underlying hypothesis was that the ongoing off-line activity during sleep in neuronal networks previously engaged in learning during wakefulness reflect the processing of recent memory traces, which eventually leads to the plastic changes underlying the subsequent improvement in performance (Maquet et al., 2003). Nonetheless, sleep represents no more than one step in the off-line course of the processing of new information, from initial encoding to consolidation in long-term memory stores. Hence, it has been proposed that part of the consolidation process could take place during wakefulness in off-line periods of behavioural inactivity that follow acquisition (Buzsaki, 1989; Hoffman and McNaughton, 2002; Marr, 1971). Still, it should be kept in mind that the awake human brain uninterruptedly processes an ongoing flow of information arising from the environment, meaning that acquisition periods are more often followed by different acquisition periods than by quiet wakefulness. Therefore, we have hypothesised that there should be a mechanism that allows the maintenance of a set of newly acquired information while facing new experiences.

Using fMRI, we found evidence that neuronal activity expressed in learning-related areas during task practice persists and evolves during the awake period that follows the end of wakefulness, and that this post-training activity can be detected even in the context of unrelated behaviours. Noteworthy, post-training persistent activity and neural reorganization at wake was shown to occur both in the context of hippocampus-dependent (i.e., spatial and declarative) and of hippocampus-independent (i.e., motor procedural) memories, suggesting that these phenomena represent fundamental mechanisms for transitory information maintenance and memory formation in the human brain.

To assess our hypothesis, 15 volunteers were scanned using event-related fMRI (3 Tesla, Siemens Allegra) while exposed to an auditory oddball task at three different times in a half-day (Figure 2). First and second scans were performed immediately before and after a 30 min episode either of spatial or procedural learning outside of the scanner. Third scan was performed after another 30 min break without practice. A fourth, block-design, session was acquired during practice of the learning task to determine the set of brain areas engaged by this task. Subjects were scanned again two weeks later under the same protocol at the same time of day but using the other learning task. Two weeks later, the same subjects were scanned again under the same protocol but using the other learning task, at the same time of day to avoid confounding circadian influences.

The hippocampus-dependent spatial memory task consisted in topographical route learning in a virtual 3D town (Maguire et al., 1998); the hippocampus-independent procedural memory task was the multiple choice serial reaction time (SRT) task (Peigneux et al., 2000), a paradigm of implicit motor sequence learning. Both spatial and procedural tasks have been shown to induce post-training neuronal activity in learning-related regions during, respectively, slow wave sleep (Peigneux et al., 2004) and rapid eye movement sleep (Maquet et al., 2000; Peigneux et al., 2003). In the auditory oddball task, subjects were merely requested to mentally count the number of deviant sounds (~30 rare events) that occurred in a monotonous flow of repeated tones (~270 frequent events). BOLD responses to the rare events in the three scanning sessions within each-half-day were the variables of interest to yield spatial- or procedural-learning related modifications of neuronal activity. BOLD data were analyzed using SPM2 using a random effect model. Results are reported at $p < .05$, small volume corrected (10 mm).

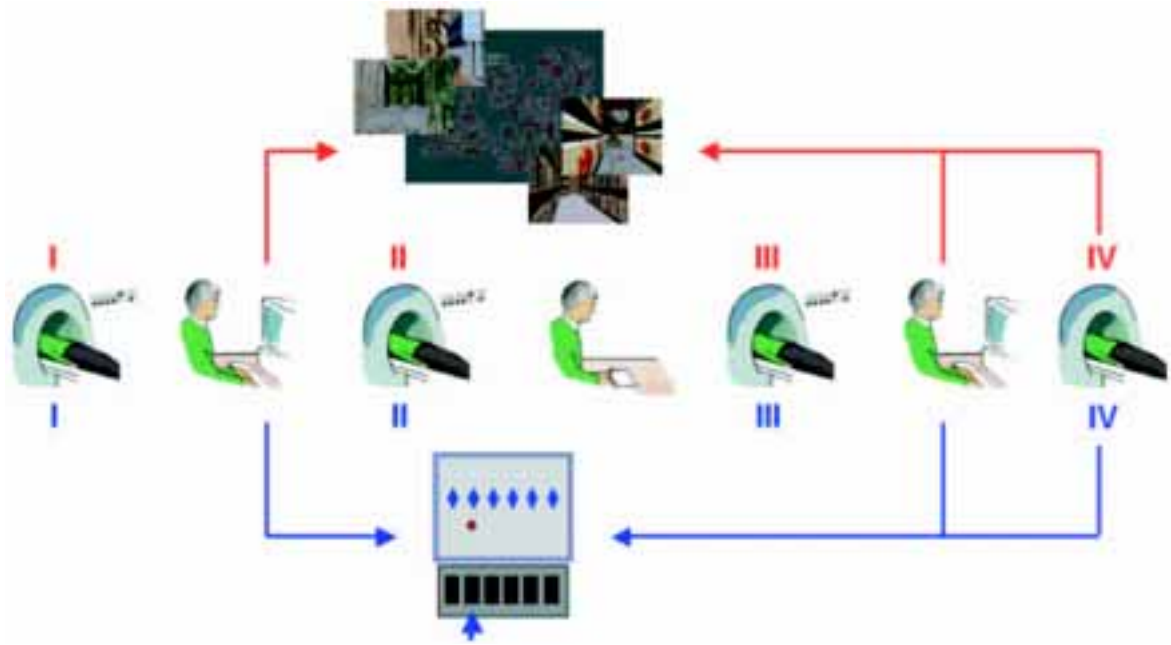

Figure 2. Experimental protocol. Subjects underwent 4 fMRI scanning sessions [I-IV] within a half-day, repeated two weeks apart. The experiment always started with auditory oddball scanning session [I] during mental counting of rare tones (duration ~8 minutes). Subjects were then trained either to the topographical/spatial memory navigation task (red path) or to the procedural memory serial reaction time (SRT) task (blue path) during ~30 minutes. Immediately after the end of learning, subjects were scanned again [II] while exposed to the auditory oddball task. They were then allowed a further 30-minutes break, during which they filled questionnaires. Then, there were scanned once again [III] while exposed to the auditory oddball task. Afterwards, subject's memory for the learned task was tested outside of the scanner. Finally, subjects underwent a fourth, bloc-design, fMRI session [IV] during which they explored virtual environments (red path) or practiced sequences in the SRT task (blue path).

Results showed that brain response to rare sounds in the oddball task was modified by prior spatial learning: higher BOLD response was found in the hippocampus (26 -24 -8 and -24 -26 -4 mm) and parahippocampal gyrus (14 -32 -6 and -16 -34 -6 mm) during oddball practice immediately after spatial learning than before, and more so than after procedural learning. Similar responses were found in a set of cortical and subcortical areas. Furthermore, activity was higher 30 min. farther during the 3rd oddball in the right parahippocampal gyrus (26 -32 -18 mm), suggesting that post-training regional cerebral activity increases off-line over time after spatial learning (Figure 3).

Conversely, prior procedural learning altered subsequent brain activity in a different way: BOLD response was higher only in the cerebellum (2 -60 -28 mm) during oddball practice immediately after procedural learning than before, and more so than after spatial learning, but did not evolve 30 minutes farther. At variance, activity in the caudate nucleus of the striatum (-16 0 18 mm) was higher during the 3rd, but not the 2nd, oddball (Figure 3). Moreover, posterior probability maps computed based on prior covariances from empirical Bayes parameters (Friston and Penny, 2003) indicated an average low probability (17 %) that activity during oddball practice II (versus I) was significant in these latter locations. Hence, in line with (Doyon et al., 2002), our results suggest that, whereas higher cerebellum activity was immediately elicited by prior training and persisted over time, activity in the striatum and other sequence-processing related cortical regions represents a second, delayed step in the off-line activity that takes place after procedural learning has ended.

Finally, psychophysiological interaction (PPI) analyses confirmed that those areas showing persistent neural activity gradually established or reinforced functional connections with other brain regions involved in learning: coupling of activity in cortical and subcortical areas with hippocampal activity was shown to increase immediately after spatial learning, whereas after procedural learning, a first, immediate increase is observed for cerebello-frontal activity, followed after a period of time of half an hour by a delayed increase in the coupling between cerebellum and striatum.

As a whole, our analyses suggest that both the offline persistence and the time-dependent reorganization of neuronal activity represent fundamental mechanisms for long-term information maintenance in the awake human brain. Neuronal activity expressed in learning-related areas persists and evolves during the awake period that follows the end of practice, and can be detected even in the context of unrelated behaviors (i.e., oddball task). This simple mechanism might initiate the cascade of wake- and sleep-related processes that further supports the consolidation of recent memories.

The manuscript summarizing these results is in preparation. The study will be also published in abstract form in : Peigneux, P., Orban, P., Schmidt, C., Balteau, E., Degueldre, C., Luxen, A., Collette, F., Maquet, P. (2005) Long-term persistent neuronal activity and information maintenance in learning-dependent cerebral networks". 11th Annual Meeting of the Organization of Human Brain Mapping, Toronto (Canada) June 12-16.

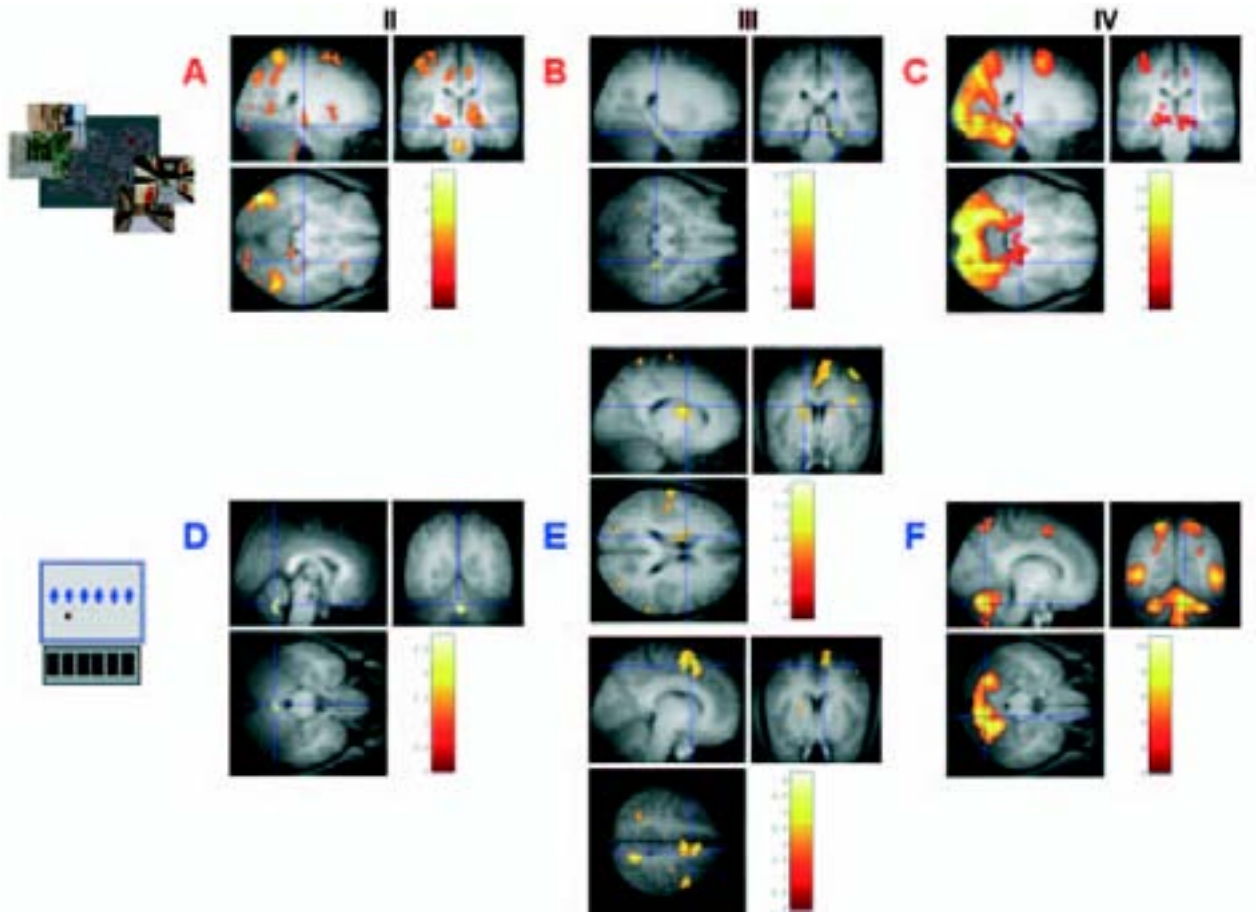

Figure 3. Persistence and reorganization of learning-related neural activity.
[A B C] spatial learning-related activity. [A] Brain areas in which BOLD response evoked by rare auditory events was higher after a 30 minutes exposure to the spatial than the procedural learning task during fMRI oddball session II (versus I). Blue hairs cross on the hippocampus (26 -24 -8 mm, $p^{svc(10mm)} < .005$) superimposed on sagittal (left top), coronal (right top) and transverse (bottom) sections of subject's average T1-weighted MRI image. Colour codes (bars) indicate magnitude of the effect size. [B] Brain areas in which BOLD response was higher after a further 30-minutes break during fMRI oddball session III (versus II). Blue hairs cross on the parahippocampal gyrus (26 -32 -18 mm, $p^{svc} < .005$). [C] BOLD response during exploration of the virtual environment in session IV. Blue hairs cross on hippocampal activation (22 -26 -6 mm, $p^{corr} < .005$).
[D E F] procedural learning-related activity. [D] Brain areas in which BOLD response evoked by rare auditory events was higher after a 30 minutes exposure to the procedural than the spatial learning task during fMRI oddball session II (versus I). Blue hairs cross on the cerebellum (2 -60 -28 mm, $p^{svc(10mm)} < .05$). [E] Brain areas in which BOLD response was higher after a further 30-minutes break during fMRI oddball session III (versus II). Blue hairs cross on the caudate nucleus (top: -16 0 18 mm, $p^{svc(10mm)} < .005$) and the pre-supplementary motor area (bottom: 10 14 58 mm, $p^{corr} < .05$). [F] BOLD response during practice of the procedural serial reaction time task in session IV. Blue hairs cross on the cerebellum (12 -74 - 22 mm, $p^{corr} < .05$) .

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3. Studies on patients with altered states of consciousness

3.1. Vegetative state

Resting brain function

We were the first to identify the functional neuroanatomy of VS patients by means of voxel based analyses of PET data. Characteristic of VS patients is a relative sparing of metabolism in the brainstem (encompassing the pedunculopontine reticular formation, the hypothalamus and the basal forebrain)(Laureys et al., 2000b). The functional preservation of these structures allows for the preserved arousal and autonomic functions in these patients. The other hallmark of the vegetative state is a systematic impairment of metabolism in the polymodal associative cortices (bilateral prefrontal regions, Broca's area, parieto-temporal and posterior parietal areas and precuneus) (Laureys et al., 1999a). These regions are known to be important in various functions that are necessary for consciousness, such as attention, memory and language (Baars et al., 2003). It is still controversial whether the observed metabolic impairment in this large cortical network reflects an irreversible structural neuronal loss, or functional and potentially reversible damage. However, in the rare cases where VS patients recover awareness of self and environment, we could show a functional recovery of metabolism in these same cortical regions (Laureys et al., 1999b). Moreover, we demonstrated that the resumption of long-range functional connectivity between these associative cortices and between some of these and the intralaminar thalamic nuclei parallels the restoration of their functional integrity (Laureys et al., 2000c). The cellular mechanisms which underlie this functional normalization remain putative: axonal sprouting, neurite outgrowth, cell division (known to occur predominantly in associative cortices in normal primates) have been proposed candidate processes (Laureys et al., 2000d). The challenge is now to identify the conditions in which, and the mechanisms by which, some vegetative patients may recover consciousness.

Brain activation studies

In our previous cohort studies of patients unequivocally meeting the clinical diagnosis of the VS, simple noxious somatosensory(Laureys et al., 2002) and auditory(Laureys et al., 2000a, Boly et al., 2004) stimuli we have shown systematic activation of primary sensory cortices and lack of activation in higher order associative cortices from which they were functionally disconnected. High intensity noxious electrical stimulation activated midbrain, contralateral thalamus and primary somatosensory cortex in each and every one of the 15 vegetative patients studied, even in the absence of detectable cortical evoked potentials (Laureys et al., 2002). However, secondary somatosensory, insular, posterior parietal and anterior cingulate cortices, which were activated in all control subjects, failed to show significant activation in a single vegetative patient. Moreover, in the VS patients, the activated primary somatosensory cortex was shown to exist as an island, functionally disconnected from higher-order associative cortices of the pain-matrix. Similarly, although simple auditory click stimuli activated bilateral primary auditory cortices in vegetative patients, hierarchically higher-order multimodal association cortices were not activated. Moreover, a cascade of functional disconnections were observed along the auditory cortical pathways, from primary auditory areas to multimodal and limbic areas (Laureys et al., 2000a) suggesting that the observed residual cortical processing in the VS does not lead to integrative processes which are thought to be necessary for awareness.

3.2. Minimally conscious state

Because criteria for the MCS have only recently been introduced, there are very few functional imaging studies of patients in this condition. Our group was the first to publish functional imaging data in this challenging patient population. Exposure to simple auditory stimulati induced a more widespread activation in minimally conscious than in vegetative patients (Boly et al., 2004). In the former, activation encompassed not only primary but also auditory associative areas, suggesting a more elaborate level of processing. Moreover, cortico-cortical functional connectivity was more efficient in the MCS, compared to the VS, between auditory cortex and a large network of temporal and prefrontal cortices. We could also show that auditory stimuli with emotional valence (infant cries and the patient's own name) induced a much more widespread activation than did meaningless noise. The activation pattern observed in MCS using $H_2{}^{15}O$-PET was comparable to that previously obtained in controls and simultaneously acquired cognitive potentials showed preserved P300 responses to the patient's own name but not to other names. Such findings encourage ongoing developments of neuromodulatory and cognitive revalidation therapeutic strategies in MCS patients (Laureys et al., 2004).

3.3. Locked-in syndrome

Classically, structural brain imaging (MRI) may show isolated lesions (bilateral infarction, haemorrhage, or tumor) of the ventral portion of the basis pontis or midbrain. According to some authors, electroencephalography (EEG) and evoked potentials do not reliably distinguish the LIS from the VS (Gutling et al., 1996). PET scanning has shown significantly higher metabolic levels in the brains of patients in a LIS compared to patients in the VS (Levy et al., 1987). Our preliminary voxel-based statistical analyses show that no supra-tentorial cortical areas show a significantly lower metabolism in LIS patients when compared to healthy controls (Laureys et al., 2003). These findings emphasize the need to quickly both make the diagnosis and recognize the terrifying situation of patients with intact awareness of self and environment in acutely locked-in immobile bodies. Heath-care workers should adapt their bedside-behaviour and consider pharmacological anxiolytic therapy, taking into account the intense emotional state acute LIS patients go through. With appropriate medical care, life expectancy may be several decades and even if the chances of motor recovery are very limited, computer-based communication methods have drastically improved the quality of life of chronic LIS patients (Laureys, 2004).

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Publications related to the project

Prizes

- 2004 : ASSC William James Prize for Contributions to the Study of Consciousness, USA

Books

- The Boundaries of Consciousness: Neurobiology and Neuropathology. Edited by Laureys S Progress in Brain Research, Elsevier, Amsterdam (under the press)
- Functional neuroimaging in hysteria and catatonia De Tiège X, Goldman S, Laureys S In: Nuclear Medicine in Psychiatry Edited by Otte A, Audenaert K, Peremans K, van Heeringen K, Dierckx RA, Springer-Verlag, Berlin 31 (2004) 521-530
- Brain function in the vegetative state Laureys S, Faymonville ME, De Tiège X, Peigneux P, Berré J, Moonen G, Goldman S, Maquet P In : Brain death and disorders of consciousness (Series title : Advances in Experimental Medicine and Biology, Edited by Machado C, Shewmon DA, Kluwer Academic/Plenum Publishers, New York, 550 (2004) 229-38
- Residual cerebral functioning in the vegetative state Laureys S, Faymonville ME, De Tiège X, Berré J, Elincx S, Antoine S, Ligot N, Boly M, Schnakers C, Sadzot B, Hansen I, Damas P, Mavroudakis N, Vincent JL, Lamy M, Moonen G, Luxen A, Goldman S, Maquet P Life-sustaing treatments and vegetative state: Scientific advances and ethical dilemmas, 17-20 March, 2004, Vatican City, Rome, Italy L Arco di Giano (2004) 74-80

Scientific papers

- Auditory processing in severely brain injured patients: differences between the minimally conscious state and the persistent vegetative state Boly M, Faymonville ME, Peigneux P, Lambermont B, Damas P, Del Fiore G, Degueldre C, Franck G, Luxen A, Lamy M, Moonen G, Maquet P, Laureys S Archives of Neurology 61 (2004) 233-238.
- Cerebral processing in the minimally conscious state Laureys S, Perrin F, Faymonville ME, Schnakers C, Boly M, Bartsch V, Majerus S, Moonen G, Maquet P Neurology 63 (2004) 916-918.
- Brain function in coma, vegetative state, and related disorders Laureys S, Owen A, Schiff N Lancet Neurology 3 (2004) 537 46
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- Cerebral processing of auditory and noxious stimuli in severely brain injured patients: differences between VS and MCS Boly M, Faymonville ME, Peigneux P, Lambermont B, Damas F, Luxen A, Lamy M, Moonen G, Maquet P, Laureys S Neuropsychological Rehabilitation, in press Brain death and the vegetative state Laureys S

Book review

- Wider than the sky: The Phenomenal Gift of Consciousness by GM Edelman. Laureys S New England Journal of Medicine (in press)
- The Vegetative State: Medical Facts, Ethical and Legal Dilemmas by B Jennett. Laureys S Advances in Clinical Neuroscience and Rehabilitation 3 (2004) 14
- Renouer avec les consciences emmurées Laureys S Nouvelles Clés 37 (2003) 46-50

Other publications

- Quelle conscience durant le coma ? Laureys S, Faymonville ME, Maquet P Pour la Science (French edition of Scientific American) 302 (2002) 122-128
- Comas: les états de conscience Laureys S, Faymonville M-E, Maquet P La Science au présent, Encyclopaedia Universalis, Paris, France, (2004) 138-141
- Wie bewusstlos ist bewusstlos? Laureys S, Faymonville ME, Maquet P Spektrum der Wissenschaft, Spezial: Bewusstsein, 1 (2004) 82-89

4. Controlled processes and automatic processes, in physiological and pathological conditions.

During the last year, we finalized four studies devoted to controlled processes and one related to automatic processing of information. The ability to shift attention between information is a major executive function, requiring controlled processes. We designed one of the very few studies presenting different shifting tasks in the same session to the same subjects. This allowed to directly compare the neural substrates of the different tasks, and to understand the precise role of selected frontal and parietal cortical regions. Self-reflection requires a control on personal information. We provide a summary of a study where we demonstrated that self-reflection is related to the activation of a precise region in the ventromedial prefrontal cortex. Noteworthy, this region is particularly lesioned in fronto-temporal dementia, a disease characterised by impaired self-reflection. Then, two clinical studies were published, demonstrating that patients with Alzheimer's disease have a decrease in controlled processes in the memory domain, while automatic responses are relatively preserved. The decrease in controlled processes is related to a reduction of brain metabolism in fronto-posterior associative cortices. Finally, we present an experiment where three different automatic processes were studied: the "simple exposure effect" (a preference related to previous exposure to a stimulus), the priming effect (a benefit for processing an information already encountered), and the familiarity effect (the feeling that a stimulus has already been seen before, without precise remembering). Simple exposure effect and priming seemed to rely on similar middle temporal brain regions, while familiarity did not.

4.1. Comparing brain activation in different shifting tasks

Shifting corresponds to the ability to regularly change the focus of attention from one to another information. Shifting is an important executive function, and there is a debate concerning unity or diversity of shifting process(es). An important cognitive study suggested that different shifting tasks shared a common process (Miyake et al, 2000). However, a recent meta-analysis showed that different brain substrates were related to different types of shifting tasks (Wager et al, 2004). We submitted young healthy volunteers to three shifting tasks in a positron emission tomography study.

Shifting between arithmetical operations was compared to two simple mathematical tasks where subjects added or subtracted "three" from a visually displayed number. Activation for the shifting task was observed in the right middle frontal cortex and the inferior frontal sulcus, probably involved in the temporal coding of the alternate shifts between operations (figure 4, left upper panel). There was also an activation in an anterior part of the inferior parietal cortex, probably involved in maintaining the alternate operations required for this shifting task in working memory.

In the second task, subject visualized pairs composed of one letter and one digit. If the pair was in the upper row of the screen, the instruction was to categorize the letter as vowel or consonant; if the pair was in the lower part of the screen, the digit had to be categorized into odd or even. The shifting task was related to bilateral activation in the posterior part of the

intraparietal sulcus (figure 4, right upper panel). A shifting task, requiring executive processes, may thus depend on parietal regions only, without involvement of the frontal lobes. Importantly, the contrast between this task and the ability to shift between mathematical operations revealed a specific activation in the medial bank of the posterior intraparietal sulcus. Such a region would be important to make shifting associations between a cue (i.e. upper or lower row on the screen) and the information to process.

In the last condition, subjects saw large geometrical figures (triangle, square, circle) made of little figures. Plain lines constituted a cue to categorize the large figure according to the number of sides. When dotted lines appeared in the picture, the little figures had to be categorized. This switching task was accompanied by an activation of the left intraparietal sulcus, extending to the left superior parietal gyrus; we already highlighted that this region is important for shifting association between cue and stimulus to process. An activation of the right precuneus was probably related to high attentional demands of high switching rate between figures (figure 4, left lower panel).

Finally, we performed a conjunction analysis between the three shifting tasks. There was an activation in the middle of the right intraparietal sulcus, probably related to amodal selective attention to behaviorally relevant information and suppression of irrelevant stimuli (figure 4, right lower panel).

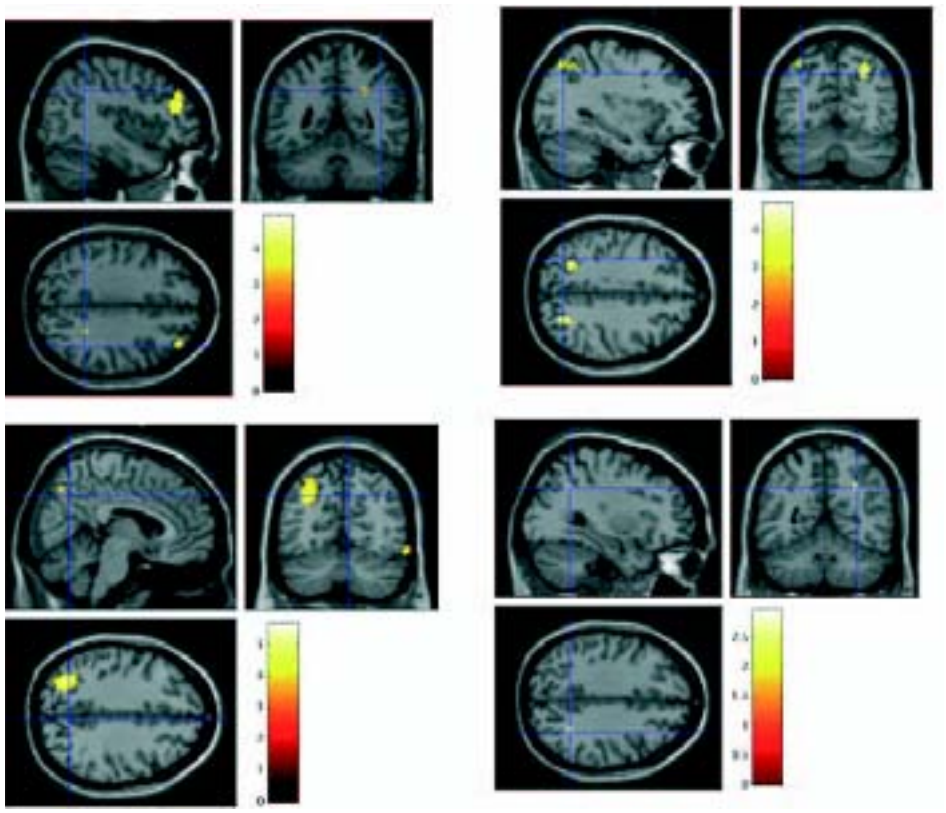

Figure 4. Left upper panel : Frontal and inferior parietal regions activated for shifting between operations
Right upper panel : The medial bank of the left intraparietal sulcus (near the blue cross) was related to shifting association between cue and information to process.
Left lower panel : Brain activation for shifting between global and local components of a figure.
Right lower panel : Common activation of the right intraparietal sulcus for the three shifting tasks.

4.2. Self-referential reflective activity and its relationship with rest: A PET study.

This study used positron emission tomography (PET) to identify the brain substrate of self-referential reflective activity and to investigate its relationship with brain areas that are active during the resting state. Thirteen healthy volunteers performed reflective tasks pertaining to three different matters (the self, another person, and social issues) while they were scanned. Rest scans were also acquired, in which subjects were asked to simply relax and not think in a systematic way. The mental activity experienced during each scan was assessed with rating scales. The results showed that, although self-referential thoughts were most frequent during the self-referential task, some self-referential reflective activity also occurred during rest. Compared to rest, performing reflective tasks was associated with increased blood flow in the dorsomedial prefrontal cortex, the left anterior middle temporal gyrus, the temporal pole bilaterally, and the right cerebellum; there was a decrease of blood flow in right prefrontal regions and in medial and right lateral parietal regions. In addition, the ventromedial prefrontal cortex (VMPFC) (1) was more active during the self-referential reflective task than during the other two reflective tasks, (2) showed common activation during rest and the self-referential task, and (3) showed a correlation between cerebral metabolism and the amount of self-referential processing (figure 5). It is suggested that the VMPFC is crucial for representing knowledge pertaining to the self and that this is an important function of the resting state, helping people to maintain a sense of personal identity across time. The paper is accepted for publication in NeuroImage.

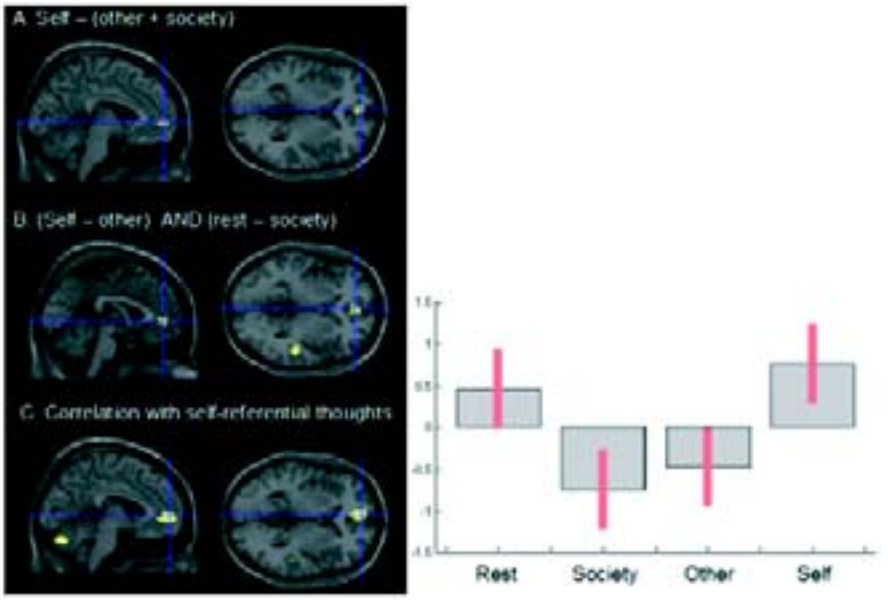

Figure 5. Left panel .Activation of the ventromedial prefrontal cortex for controlled self-reflection
Right panel. Relative activation of the ventromedial prefrontal cortex for both self reflection and rest

4.2. Exploration of controlled and automatic memory processes in early Alzheimer's disease

The aim of this study was to explore automatic and controlled processes in Alzheimer's disease (AD) by using a variant of the word-stem completion task that applies the process-dissociation procedure (PDP). Several methodological precautions were taken in order to limit problems observed in previous studies (e.g., poor task sensitivity, ceiling and/or floor effects, no control over comprehension of instructions, etc.). Our results: (1) confirmed the marked deterioration in controlled processes; (2) showed that, insofar as psychometric constraints were limited, automatic memory processes were preserved in AD. These data are in line with more global studies suggesting that AD is characterized by an early deterioration in controlled processes and an initial preservation of automatic processes. The paper is now accepted in Neuropsychology.

4.3. Cerebral metabolic correlates of four dementia scales in Alzheimer's disease

Different scales can be used to evaluate dementia severity in Alzheimer's disease (AD). They do assess different cognitive or functional abilities, but their global scores are frequently in mutual correlation. Functional imaging provides an objective method for the staging of dementia severity. Positron emission tomography was used to emphasize the relationship between brain metabolism and four dementia scales that reflect patient's global cognitive abilities (mini mental state), caregiver's evaluation of cognitive impairment (newly designed scale), daily living functioning (instrumental activities of daily living) and global dementia (clinical dementia rating). We wondered if different clinical dementia scales would be related to severity of metabolic impairment in the same brain regions, and might reflect impairment of common cognitive processes. 225 patients with probable AD were recruited. All clinical scales were related to brain metabolism in associative temporal, parietal or frontal areas. A factorial analysis demonstrated that all scales could be classified in a single factor. That factor was highly correlated to decrease of cerebral activity in bilateral parietal and temporal cortices, precuneus, and left middle frontal gyrus (figure 6). This result suggests that global scores for all scales provided similar information on the neural substrate of dementia severity. Capitalizing on the neuroimaging literature, dementia severity reflected by reduced metabolism in posterior and frontal associative areas in AD might be related to a decrease of controlled processes. The paper is accepted in the Journal of Neurology.

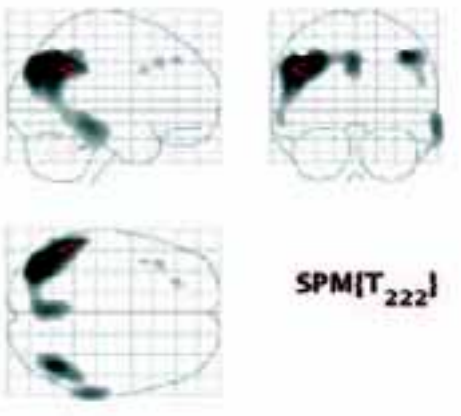

Figure 6. Correlation between a factor common to four dementia scales and brain metabolism in associative cortices of Alzheimer patients

4.4. Neural mechanisms of mere exposure effect: a comparison with priming and familiarity effects

The mere exposure effect (MEE) corresponds to the increase in affective preference toward particular stimuli that results from previous repeated exposure to those stimuli. Some authors have proposed that the MEE belongs to the same category of implicit memory phenomena as perceptual priming and familiarity-based recognition. At this time, only one study explored the neural substrates of MEE (Elliott & Dolan, 1998) and showed that MEE was associated to right lateral prefrontal activation, contrary to explicit recognition that recruited left frontopolar and parietal areas. However, due to the procedure used, these results are not easily comparable to other functional imaging studies on priming or familiarity-based recognition effects. Consequently, we aimed to explore with fMRI cerebral areas associated to priming, MEE and familiarity-based recognition effects using a same material (possible and impossible 3D objects) and task format (yes/no judgement).

Forty-eight right-handed healthy volunteers (age range: 20-26 years; twenty-four females) took part in the experiment. During the encoding phase, 42 non-familiar 3D objects (possible and impossible) were randomly presented four times and subjects were asked various perceptual judgments. During the test phase, these objects were presented mixed to new ones and subjects had to quickly perform yes/no judgment tasks. Three groups were constituted, varying according to the judgment required: preference (is the object liked or not?), object decision (is the figure possible or impossible?) and familiarity (was the object already presented?) Data were acquired on a 3T scanner using a T2* sensitive gradient echo (EPI) sequence (TR=2130msec, TE=, 40msec, 32 contiguous 3-mm thick transverse slices). In each session 600 functional volumes were obtained. Data were analyzed using SPM2 and a random effects model at the group level. An event-related model was used, with the three group of subjects processed separately and the four kind of items (old and new, possible and impossible) processed simultaneously.

Behavioural data indicated significant MEE and familiarity for possible and impossible objects but priming effect for possible objects only (all ps<0.05). The comparison of old to new items demonstrated increase of activity in the middle temporal gyrus bilaterally for the MEE effect and left-sided for the priming effect. Recognition effect was only associated with increased activity in the left middle frontal gyrus.

First, these results indicate that repeated exposure effect in preference and priming tasks can be associated to activation of common cerebral areas. Indeed, a repetition enhancement effect was found in the middle temporal gyrus. These data did not replicate the broadly observed 'repetition suppression effects' of priming (i.e., a decrease of activity). Repetition suppression is therefore not an automatic consequence of perceptual processing of stimuli. Second, the temporal region associated with MEE and priming was not similar to that found for recognition effect. These data suggest that processes that subserved priming and MEE did not support familiarity-driven recognition judgments in the context of this experiment.

Publications 2004

- Further exploration of controlled and automatic memory processes in early Alzheimer's disease
  Adam, S., Van der Linden, M., Collette, F., Lemauvais, L., Salmon, E.
  Neuropsychology 2004 (accepted)
- Modulation of Brain Activity during Phonological Familiarization
  Steve Majerus, Martial Van der Linden, Fabienne Collette, Steve
  Laureys, Martine Poncelet, Christian Degueldre, Guy Delfiore, André Luxen, Eric Salmon
  Brain and Language 2004 (accepted)
- Memory evaluation with a new cued recall test in patients with mild cognitive impairment and Alzheimer's disease
  Adrian Ivanoiu, Stephane Adam, Martial Van der Linden, Eric Salmon, Anne-Claude Juillerat, Reinhild Mulligan, Xavier Seron
  J Neurol 2004 (accepted)
- Anosognosia in very mild Alzheimer´s disease but not in mild cognitive impairment.
  Kalbe E, Salmon E, Perani D, Holthoff V, Sorbi S, Elsner A, et al.
  Dement Geriatr Cogn Disord 2004 (in press)
- Cerebral metabolic correlates of four dementia scales in Alzheimer's disease
  E. Salmon, S. Lespagnard, P. Marique, F. Péters, K. Herholz, D. Perani, V. Holthoff, E. Kalbe, D. Anchisi, S. Adam, F. Collette, G. Garraux
  J Neurol 2004 (accepted)
- Imaging a cognitive model of apraxia: the neural substrate of gesture-specific cognitive processes
  Peigneux P, Van der Linden M, Garraux G, Laureys S, Degueldre C, Aerts J, Del Fiore G, Moonen G, Luxen A, Salmon E
  Hum Brain Mapp 2004, 21(3):119-42
- Variability in the impairment of recognition memory in patients with frontal lobe lesions
  Bastin C, Van der Linden M, Lekeu F, Andrès P, Salmon E
  Cortex 2004 (accepted)
- Self-referential reflective activity and its relationship with resting consciousness: a PET study.
  D'Argembeau A., Collette F., Van der Linden M., Laureys S., Del Fiore G., Degueldre C., Luxen A., Salmon E.
  Neuroimage 2004 (accepted)
- Exploring the unity and diversity of the neural substrates of executive functioning.
  Collette F., Van der Linden M., Laureys S., Del Fiore G., Degueldre C., Delfiore G., Luxen A., Salmon E.
  Human Brain Mapping 2004 (accepted)

Final Report of the Research Group of

Prof. Dr. Moonen G.

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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 or 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 CRD-NRG signalling 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 (CRD-ICD) and the CRD-NRG itself. Then, we have purified both of them we raised a rabbit polyclonal antiserum against CRD-ICD and monoclonal antibodies against CRD-NRG. We could obtain five monoclonal antibodies and we are now checking which their respective epitope are. Using purified IgG from the polyclonal serum against CRD-ICD, we observed in immunocytofluorescent studies that CRD-ICD is present at 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 CRD-NRG and the CRD-ICD is then transferred to nucleus. This nuclear translocation is followed by a oligodendroglial or a neuronal differentiation. It has been recently demonstrated that CRD-NRG is cleaved at the membrane level (Frenzel and Falls, 2001). Moreover, the CRD-ICD has been described in the nucleus of neurons (Bao et al., 2003). So it is important to look for the protein(s) interacting with CRD-ICD before the translocation into the nucleus. We designed a TAP-TAG approach and we could purify four interacting CRD-ICD interacting proteins from NP. These proteins will be identified by MALDI-TOF.

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. The expressed NRG-2 isoforms is related to two already described isoforms (Busfield et al., 1997; Higashiyama et al., 1997). In intact embryonic striata, a transmembrane isoform is expressed. Proliferating NP expressed in culture both an transmembrane and a secreted form of NRG-2.

The expression of these two isoforms dramatically decrease in differentiating NP. We have cloned the secreted NRG-2 isoform and we are expressing it. We are now using this form in NP cultures during the proliferation or the differentiation process. Finally, we are now using polyclonal antibody against NRG-2 in culture in order to inhibit the endogenous NRG-2 and in western blot and immunofluorescence studies.

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Extrinsic and intrinsic regulation of proliferation and fate specification in the oligodendroglial lineage

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

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 $5\text{-}HT_3Rs$ in other experimental paradigms of oligodendroglial differentiation in vitro and notably T3-induced lineage progression, iii) the intracellular pathway(s) linking $5\text{-}HT_3R$ 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.

Finally, cells that express the NG2 chondroitin sulphate proteoglycan (NG2) represent the largest pool of postnatal/adult proliferative progenitors in the brain (Dawson et al., 2000). These progenitors are found both in non-neurogenic, as well as neurogenic areas of the CNS. NG2-expressing cells were thought to be strictly oligodendrocyte progenitors, however our recent studies have expanded their role by demonstrating that $NG2^+$ cells form neurospheres and generate neurons in vitro (Belachew et al., 2003). The neurogenic properties of $NG2^+$ cells were demonstrated by using a 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP)-EGFP transgenic mouse, in which $NG2^+$ progenitors expressed CNP-promoter-driven EGFP (Yuan et al., 2002; Belachew et al., 2003). Consistent with these results, a parallel study demonstrated that $A2B5^+$ precursor cells isolated from the adult human subcortical white matter expressed the CNP gene and generated neurons in vitro and in vivo (Nunes et al., 2003). Given these findings, it is clear that CNP gene-expressing cells that are NG2 and A2B5 immunopositive display properties of multipotent progenitor cells (Belachew et al., 2003; Nunes et al., 2003; Goldman, 2003). It is therefore possible that these cells could contribute to neurogenesis in the postnatal/adult brain, although the precise characterization and neurogenic potential of $NG2^+$ progenitors in the SVZ and the classification of the neuronal subtypes that they generate remain undefined.

We have previously examined the properties of $NG2^+$ precursor cells in the CNP-EGFP transgenic mouse, because in this mouse strain all $NG2^+$ cells in the SVZ express EGFP (Yuan et al., 2002; Belachew et al., 2003). Furthermore, we have recently demonstrated that $NG2^+/EGFP^+$ cells in the SVZ are mitotically active, and express proposed markers of multipotent precursor cells in the SVZ (Dlx, EGFR and LeX) (Aguirre et al., 2004). Based on the expression of these markers, we have established a relationship between $NG2^+$ and LeX^+ precursor cells in the SVZ, and

determined a lineage continuum between $NG2^+/Dlx^+$ progenitors and endogenous hippocampal GABAergic neurons (Aguirre et al., 2004). To determine whether $NG2^+$ cells can contribute to neurogenesis in vivo, we double FACS-purified $NG2^+/EGFP^+$ cells from CNP-EGFP mice and isochronically transplanted these cells into the lateral ventricle of wild-type mice (Aguirre et al., 2004). We demonstrated that engrafted $NG2^+/EGFP^+$ progenitors migrated to the hippocampus, where they gave rise to Dlx^+ GABAergic-interneurons (Aguirre et al., 2004). Our findings indicated that $NG2^+$/CNP gene-expressing cells are the Lex^+/GFAP-negative Type C cells in the SVZ, and for the first time identify Type C-like cells as a source of hippocampal GABAergic interneurons in the postnatal brain (Aguirre et al., 2004). The possibility of obtaining a viable and highly purified population of endogenous neural progenitors and to direct the generation of GABAergic neurons from these precursors could have very important implications for future therapeutic approaches to epilepsy, stroke and degenerative damage of the brain.

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Phenotypic plasticity of radial glial cells in vitro and in vivo: Comparison of the proteomes of the radial glial cells and astrocytes.

Leprince, P. and Moonen, G.

The transformation of radial glia into astrocytes, which normally occurs at the end of the period of neuronal migration, appears to be a reversible phenomenon since the transformation of astrocytes into more immature cells can be induced in the adult brain (Hunter and Hatten, 1995). To better understand the molecular mechanisms which control this bidirectional transition is a prerequisite if one wants to manipulate the phenotype of astrocytes in the adult brain in the case of graft of neuroblasts. Important information on this process of phenotypical conversion, sometimes described under the term of transdifferentiation, could be obtained by the analysis and comparison of the proteomes of the radial glia, astrocytes as well as other phenotypes that can be produced in vitro under specific culture conditions. To this end, we implement the technique of two-dimensional electrophoresis based on the new methodology of 2D DIGE which allows a comparative analysis of proteomes with an unequalled precision (Tonge et al., 2001). As it is necessary, in this approach, to use as pure as possible cell cultures for the analysis, almost homogeneous initial populations must be obtained at various ages of development. We thus developed a culture protocol for cerebellar radial glia from mouse embryo in a serum-free medium enriched in growth factors which allows the almost complete maintenance of the phenotype of stem cells : intense proliferation, expression of nestin, growth in neurosphere, absence of GFAP expression. These cells are compared with purified postnatal cerebellar astrocytes which express GFAP and have a stellar morphology. We now have all the necessary equipment to implement the 2D-DIGE technique that will be used to study the expression of proteins by the various cell types. Those proteins that will show a differential expression will be prepared in parallel gels loaded with greater quantities of material and colored by the Ruthenium method. These protein spots will be subjected to mass spectrometry sequencing in collaboration with Pr. E De Pauw.

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Study of the effect of Reelin on the morphology and biochemical maturation of radial glia
Leprince P.

We took part in an international research project aiming at determining which effect Reelin, a protein of the extracellular matrix present in the cortex in development, has on the morphology and the biochemical maturation of radial glia. Reelin is in addition known for its implication in the control of neuronal migration and the definition of the cortical layers. We showed that Reelin controls the production of radial fibre by radial glial cells and their expression of BLBP, an important protein for the control of neuronal migration. This role is observed in the cortex but not in the basal ganglia. It occurs as a direct effect of Reeline on the radial glia which implies a signaling mechanism using the adaptator protein Dab1. This work leads to the publication of an article in 2003 (Hartfuss et al., 2003).

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Study of the mode of action of Levetiracetam, a new antiepileptic drug.
Leprince P, Rigo JM, Hans G, Nguyen L, Rocher V, Belachew S, Malgrange B, Moonen G

Our laboratory collaborated during several years with a team at UCB for the characterization of the mode of action of Levetiracetam (Keppra), a new antiepileptic drug. In this context we took part in the study of the action of levetiracetam on the inhibitory neurotransmission. This work allowed to show inhibition by levetiracetam of the effect of negative allosteric modulators of the currents induced by GABA and glycine in cultures of neurons (Rigo et al., 2002). We in addition carried out many measurements of the parameters characterizing the interaction of tritiated UCB 30889, a radioactive analogue of levetiracetam, with its binding sites on cultures of nerve cells. The dissociation constant (Kd) and numbers of binding sites (Bmax) were determined with cultures of cortical neurons, cerebellar neurons and astrocytes of mouse cortices obtained during the perinatal period. An article that details these results was published in 2003 (Fuks et al., 2003).

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Cell biology for prevention and treatment of sensorineural deafness

Malgrange, B., Lallemend, F., Lefebvre, P.P., Belachew, S., Nguyen, L., Thiry, M. and Moonen, G.

Most hearing loss results from lesions of the sensory cells and/or of the neurons of the auditory part of the inner ear. There is currently no treatment able to stop the progression of a hearing loss or to restore a lost auditory function. Our work focuses on the prevention of cell death or on the induction of regeneration of the hair cells and of the auditory neurons in the cochlea.

1. The sensory compartment

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) and HC production never occurs at later stages in normal conditions. To obtain a production of new 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. 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 (Malgrange et al., 2003).

2. The neuronal compartment

Primary afferent auditory neurons reside in the spiral ganglion (SG) and are bipolar neurons that extend a peripheral process to the hair cells in the organ of Corti and a central process to the cochlear nucleus. Degeneration of the SG Neurons (SGNs) occurs secondary to the loss of the HCs, thus increasing the functional impairment. Efforts to maintain neuronal integrity constitute an important strategy in the development of pharmacological therapies for hearing recovery. Here, we have investigated the ability of substance P (SP) to protect 3-day-old (P3) rat SGNs from trophic factor deprivation (TFD)-induced cell death. The presence of SP high affinity neurokinin-1 receptor (NK1) transcripts was detected in the spiral ganglion and the NK1 protein localized to SGNs both ex vivo and in vitro. Treatment with SP increased cytoplasmic Ca2+ in SGNs, further arguing for the presence of functional NK1 on these neurons. Both SP and the agonist [Sar9,Met(O2)11]-SP significantly decreased SGN cell death induced by TFD, with no effect on neurite outgrowth. The survival promoting effect of SP was blocked by the NK1 antagonist, WIN51708. Both pancaspase inhibitor BOC-D-FMK and SP treatments markedly reduced activation of caspases and DNA fragmentation in trophic factor deprived-neurons. The neuroprotective action of SP was antagonised by specific inhibitors of second messengers, including 1.2-bis-(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM) to chelate cytosolic Ca2+, the protein kinase C (PKC) inhibitors bisindolylmaleimide I, Go¨6976 and LY333531 and the MAPK/ERK inhibitor U0126. In contrast, nifedipine, a specific inhibitor of L-type Ca2+ channel, and LY294002, a phosphatidylinositol-3-OH kinase (PI3K) inhibitor, had no effect on SP trophic support of SGNs. Moreover, activation of endogenous PKC by 4b-phorbol 12-myristate 13-acetate (PMA) also reduced the loss of trophic factor-deprived SGNs. Thus, NK1 expressed by SGNs transmit a survival-promoting regulatory signal during TFDinduced SGN cell death via pathways involving PKC activation, Ca2+ signalling and MAPK/ERK activation, which can be accounted for by an inhibition of caspase activation (Lallemend et al., 2003).

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Neurotransmitters as developmental signals

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

1) Autocrine/paracrine activation of the $GABA_A$ receptor inhibits the proliferation of neurogenic PSA-NCAM+ precursor cells from postnatal striatum

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 $GABA_AR$ 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.

2) Kinetics properties of the alpha2 homo-oligomeric glycine receptor impairs a proper synaptic functioning (Journal of Physiology, 553 (2003) 369-386)

Ionotropic glycine receptors (GlyRs) are present in the central nervous system well before the establishment of synaptic contacts. Immature nerve cells are known, at least in the spinal cord, to express α2 homomeric GlyRs, the properties of which are relatively unknown compared to those of the adult synaptic form of the GlyR (mainly α1/β heteromeres). Here, the kinetics properties of GlyRs at the single-channel level have been recorded in real-time by means of the patch-clamp technique in the outside-out configuration coupled with an ultra-fast flow application system (< 100 µs). Recordings were performed on CHO cells stably transfected with the α2 GlyR subunit. We show that the onset, the relaxation and the desensitization of α2 homomeric GlyR-mediated currents are slower by one or two order of magnitude compared to synaptic mature GlyRs and to other ligand-gated ionotropic channels involved in fast synaptic transmission. First latency analysis performed on single GlyR channels revealed that their slow activation time-course was due to delayed openings. When synaptic release of glycine was mimicked (1 mM glycine; 1 ms pulse duration), the opening probability of α2 homomeric GlyRs was low (Po $\approx$ 0.1) when compared to mature synaptic GlyRs (Po = 0.9) (Legendre, 1998). This low Po is likely to be a direct consequence of the relatively slow activation kinetics of α2 homomeric GlyRs when compared to the activation kinetics of mature α1/β GlyRs. Such slow kinetics suggest that embryonic α2 homomeric GlyRs cannot be activated by fast neurotransmitter release at mature synapses but rather could be suited for a non-synaptic paracrine-like release of agonist, which is known to occur in the embryo.

3) Striatal PSA-NCAM+ precursor cells from newborn rat express functional strychnine-sensitive glycine receptors (NeuroReport, 15 (2004) 583-587)

In this work, we have assessed the presence of ionotropic glycine receptors in neurogenic progenitors purified from the newborn rat striatum and expressing the polysialylated form of the neural cell adhesion molecule. Using immunocytochemical analysis, we report that glycine receptors are expressed in these cells at the ßIII-tubulin expressing stage, both *in vitro* and *in situ*. To ascertain whether glycine receptors were functional *in vitro*, whole-cell patch-clamp recordings demonstrated that glycine triggers inward strychnine-sensitive currents in the majority of cultured striatal neurogenic progenitors that express the polysialylated form of the neural cell adhesion molecule. Glycine-evoked currents were completely and reversibly inhibited in a concentration-dependent manner by the glycine receptor antagonist strychnine. Moreover, we found that glycine receptors expressed by these neurogenic progenitors display intermediate electrophysiological characteristics between those of glycine receptors expressed by neural stem cells and by mature interneurons from the rat striatum. Altogether, the present data show that functional strychnine-sensitive glycine receptors are expressed in neurogenic progenitors purified from the newborn rat striatum.

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Prevention of neuronal cell death

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

1) ß-Carbolines induce apoptosis in cultured cerebellar granule neurons via the mitochondrial pathway.

N-butyl-ß-carboline-3-carboxylate?(ßCCB) is, together with 2-methyl-norharmanium and 2,9-dimethylnorharmanium ions, an endogenously occurring ß-carboline. Due to their structural similarities with the synthetic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), harman and norharman compounds have been proposed to be involved in the pathogenesis of Parkinson's disease. While also structurally related, ßCCB has received much less interest in that respect although we had previously demonstrated that ßCCB induces the apoptotic cell death of cultured cerebellar granule neurons (CGNs). Herein, we have investigated the molecular events leading to CGN apoptosis upon ßCCB treatment. We first demonstrated that ßCCB-induced apoptosis occurs in neurons only, most likely as a consequence of a specific neuronal uptake as shown using binding/uptake experiments. Then we observed that, in ßCCB-treated CGNs, caspases 9, 3 and 8 were successively activated, suggesting an activation of the mitochondrial pathway. Consistently, ßCCB also induced the release from the mitochondrial intermembrane space of two pro-apoptotic factors, i.e. cytochrome c and apotptosis inducing factor (AIF). Interestingly, no mitochondrial membrane depolarisation was associated with this release, suggesting a mitochondrial permeability transition pore-independent mechanism. The absence of any neuroprotective effect provided by two mPTP inhibitors, i.e. cyclosporine A and bongkrekic acid, further supported this hypothesis. Together, these results show that ßCCB is specifically taken up by neuronal cells where it triggers a specific permeabilization of the outer mitochondrial membrane and a subsequent apoptotic cell death.

2) Peripheral benzodiazepine receptor-independent (PBR) ligands-induced cell death

Some synthetic ligands of the peripheral-type benzodiazepine receptor (PBR), an 18 kDa protein of the outer mitochondrial membrane, are cytotoxic for several tumor cell lines and arise as promising chemotherapeutic candidates. However, conflicting results were reported regarding the actual effect of these drugs on cellular survival ranging from protection to toxicity. Moreover, the concentrations needed to observe such a toxicity were usually high, far above the affinity range for their receptor, hence questioning its specificity. In the present study, we have shown that micromolar concentrations of FGIN-1-27 and Ro 5-4864, two chemically unrelated PBR ligands are toxic for both PBR-expressing SK-N-BE neuroblastoma cells and PBR-deficient Jurkat lymphoma cells. We have thereby demonstrated that the cytotoxicity of these drugs is unrelated to their PBR-binding activity. Moreover, Ro 5-4864-induced cell death differed strikingly between both cell types, being apoptotic in Jurkat cells while necrotic in SK-N-BE cells. Again, this did not seem to be related to PBR expression since Ro 5-4864-induced death of PBR-transfected Jurkat cells remained apoptotic. Taken together, our results show that PBR is unlikely to mediate all the effects of these PBR ligands. They however confirm that some of these ligands are very effective cytotoxic drugs towards various cancer cells, even for reputed chemoresistant tumors such as neuroblastoma, and, surprisingly, also for PBR-lacking tumor cells.

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Development of Proteomic analysis by two dimensional electrophoresis using the technique of 2D-DIGE

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We have used for several years the technique of two dimensional electrophoresis of proteins to analyse the expression of proteins and to identify them in a broad range of samples. Thanks to the assistance of the FNRS and the Special Funds for Research in the Universities, we could equip our laboratory with equipment, reagents and programs for data processing in order to create the first platform of two dimensional electrophoresis by 2D-DIGE in the French Community. The technique of 2D-DIGE allows the quantitative analysis of differences in protein expression at the level of whole proteome with an unequalled precision and a statistical validation of the results. This technology was started at the end of 2003 in our laboratory and is already applied to some of our research projects and to other external collaborations which come to use our competence and our resources in this field: Our competence in the analysis of proteins by two dimensional electrophoresis was used within the framework of a collaboration with Dr. Thomas and Prof Mainil of the service of bacteriology of the Faculty of Veterinary Medecine. This study aims at identifying differences in expression of adhesins, the proteins of adhesion of Mycoplasma bovis, between strains of these pathogenic bacteria selected for their low or high adherence to cells of the host. Three membrane proteins strongly expressed by the adherent strains were identified and proved to be new forms of Vsp. An immune serum prepared against these proteins strongly reduces the adherence of Mr. Bovis to the host cells, confirming the importance of these proteins in the pathogenic process. This work was the subject of an article in press (Thomas et al., 2005).

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Study of the localization of thiamin triphosphatase in the brain of the rodents

Czerniecki J, Chanas G, Verlaet M, Bettendorff L, Makarchikov AF, Leprince P, Wins P, Grisar T, Lakaye B

We took part in a study by in situ hybridization and immunostaining of the distribution of Thiamin triphosphatase of 25 kDa, an enzyme implied in the control of the concentration of Thiamin triphosphate, the neuroactive form of this vitamin. Thiamin triphosphate is a phosphate donor for certain proteins and could belong to a new system of intracellular signal transduction. The enzyme was identified in several neuronal populations as well as in astrocytes. Its distribution in dendrites and cell bodies suggests a general role in the neuronal metabolism rather than a specific role in exitability. This work lead to the publication of an article in 2004 (Czerniecki et al., 2004).

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

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Recently, several observations report that adult somatic stem cells are not tissue-specific but exhibit a phenotypic plasticity. By example, when mesenchymal stem cells (MSC) 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 (Brazelton et coll., 2000; Mezey et coll., 2000).

We have established the culture method to isolate and cultivate MSC 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 MSC could differentiate into adipocytes, osteocytes and chondrocytes. In specific culture conditions, they become able to express the nestin, an intermediate filament protein which is highly expressed in immature cells of the nervous system. The nestin expression is only observed MSC cultures which are old enough in culture after serum withdrawal: before ten passages (25 doubling cell populations), no nestin could be seen in favourable culture conditions. It means that MSC 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 MSC are able to expressed nestin, they can form clusters in non-adherent culture conditions, just like neural stem cells do.

When nestin(+) but not nestin(-) MSC 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. It was recently reported that in vivo MSCs are able to fuse with neurons (Weinmann et al., 2003; Alavarez-Dolado et l., 2003).We demonstrated using two procedures that those GFAP(+) cells are from stromal origin and are not a consequence of a cell fusion between a MSC and a neural stem cell. Finally, nestin(-) MSC 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(+) MSCs (Wislet-Gendebien et al., 2003).

More recently, we started to co-cultivate nestin(+)MSCs with granule cerebellar neurons and in those conditions, we could observe that neuron-like cells differentiate from MSCs: they loks like neurons in culture, they express the specific neuronal markers TuJ1, NeuN, MAP2ab, Neurofilament proteins, synaptobrevin, …. . We do not rule out the possibility of cell fusion in our co-culture but we also demonstrate that the neuron-like cells differentiate from cultured MSCs and are not result of cell fusion with co-cultivated granule cerebellar neurons (Wislet-gendebien et al., 2005). Indeed, when nestine(+) MSCs are co-cultivated with paraformaldehyde-fixed neurons, they are able to differentiate into NeuN(+) neuron-like cells. Finally, electrophysiological recordings allow us to observe the successive expression of potassium voltage-gated channels, then sodium voltage-gated channels. Those cells are also able to produce isolated action potentials and to respond to the application of various neurotransmitters (GABA, glycine, serotonin and glutamate). In conclusion, the functional evaluation of these MSCs-derived neurons-like cells demonstrates that those cells behave exactly as neurons. Finally, we demonstrated that nestin(+) MSC release biologically-active BMP4 in their culture medium which in turn induces an astrocytic differentiation in co-cultivated neural stem cells (Wislet-Gendebien et al., 2004).

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Final Report of the Research Group of

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The main purpose of this grant was to support the work of a young PhD student Santosh Mysore Gopalrao, who studies the processing of kinetic and other dynamic boundaries in V4 of the monkey.

Experiment 1.

The first two years were devoted to the study of V4 neuron responses to static kinetic boundaries, either kinetic gratings or kinetic shapes. Both types of stimuli have been used in our earlier single-cell studies of IT, MT/V5, V1 and V2 (Sáry et al. Science 1993: Marcar et al. J. Neurophysiol. 1995; Marcar et al. J. Neurophysiol. 2000).

Cells to be considered selective for kinetic gratings had to give significant responses and be selective for orientation of parallel and orthogonal kinetic gratings and to prefer the same orientation of these two types of kinetic gratings. Out of a total of 482 neurons recorded in central and dorsal V4 of two monkeys 52 neurons (11%) were selective for kinetic gratings. Most of these neurons were also tuned to orientation of luminance gratings and had similar preferred orientation for the luminance and kinetic gratings. Similar results were obtained for the kinetic shape stimuli: 63 out of 295 V4 neurons tested were selective for the kinetic shape. Again most of them were also selective for luminance defined shapes and the preferred shapes for the two cues (motion and luminance difference) generally matched. Thus the cue invariant coding of shape we observed earlier in infero-temporal (IT) cortex (Sáry et al. 1993), actually starts already in the V4, which provides the dominant input to IT.

These single V4 cells were also tested with uniformly translating random textured patterns and transparent motion patterns. Average response levels and time courses of responses were compared to those obtained with kinetic and luminance gratings. V4 neurons responded more to luminance than to kinetic gratings, but less to motion and transparent motion than to kinetic patterns.

Comparison single cells and fMRI.

One of the objectives of this work was to compare the single-cell results, considered as ground truth, with the fMRI signals obtained with similar stimuli. This comparison was performed during the third year. We had obtained data from three monkeys (different from those recorded from) in which we had contrasted kinetic gratings to the control condition transparent motion, which was also tested on all V4 neurons (see above).The main result is that in all three monkeys we observed a significant activation in V4 when comparing kinetic gratings to transparent motion, but only in restricted small patches a few mm across. This is reminiscent of the single-cell studies were the selective neurons tended to be clustered. These patches had a consistent localization within each monkey for the repeated testing of the same contrast in data obtained in different experimental sessions. From this comparison we may infer that 10% selective neurons can be detected with fMRI, provided one use the local maximum approach such as SPM. The signals are too weak for an ROI approach to yield significant results.

Experiment 2.

The other objective of the work was to compare neuronal responses to the static kinetic gratings and to other types of dynamic boundaries. This was also pursued the third year. There are many ways in which dynamic boundaries can be created (using differences in motion). Ecologically the most important type of dynamic contour is one created by the displacement of a moving object. Thus we created shapes in which both the envelop and the dots inside the envelop moved (in our classical kinetic stimuli the envelop remained stationary). To understand the possible differences between these 'moving dynamic shapes' and our standard kinetic shapes (in both stimuli shape vanishes when the motion stops), we also tested static and moving luminance defined shapes, as well as static shapes created by motion of the dots inside the envelop. Further tests then investigated the effect of background and envelop motion.

So far we have tested over 70 neurons (one monkey). Preliminary analysis shows that V4 neurons keep their shape selectivity over all the stimulus manipulations. There is a tendency for the 'moving dynamic shapes' to produce more selective responses than the classical kinetic shapes.

In these experiments the time course of the presentation was different from that in the first experiment and static luminance shapes were presented for a much longer time (3-4 sec) compared to 600 ms in the original experiment. As a consequence the response of V4 neurons to luminance gratings (which evoke a strong initial transient at onset) was now smaller than that to kinetic gratings (which evoke no transient at onset), as was observed in the fMRI. This shows that when comparing fMRI and single cells the time course of stimulus presentation, is important, especially when cells show transients at stimulus onset.

Publications

Only in abstract form

- MYSORE S.G., RAIGUEL S., VOGELS R. and ORBAN G.A. Processing of kinetic boundaries in macaque V4. Soc. Neurosci. Abstr., 2003, 439.5. (33rd Society for Neuroscience Meeting, New Orleans, November 8-12, 2003)

Final Report of the Research Group of

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Activities on the analysis of the primary function of frataxin, on the development of cellular models and on a cellular therapy of Friedreich ataxia (FA) continued during the third year of FMRE support.

Two graduate students and a postdoc work on the project. One of the graduate students is paid on FMRE funds.

Pathogenesis of cellular dysfunction and death in frataxin deficiency

Overall, this project has suffered from delays following the PI's move from Montréal to Brussels and the establishment of an entirely new laboratory and research group in Brussels. Thanks to the acquisition of adequate lab space, which unfortunately was fully available only early in 2004, and of the continued grant support from FMRE and other agencies, the research group is now fully operational.

Main progress on 2004 involved:

1. The generation of cellular models and two new mouse models for FA.

The first one is a knock-in model with a modified inducible frataxin promoter using the Tet-on system. We made a construct containing the mouse frataxin promoter, then the Tet operator followed by the mouse frataxin cDNA and a polyA sequence different from the endogene (in order to distinguish the messenger from the endogene versus the transgene). This construct was used for homologous recombination in mouse ES cells carrying a deleted HPRT gene. Our construct contains a functional HPRT gene that, after homologous recombination, can restore HPRT function. Clones were selected on a HAT medium for their capacity to restore the HPRT function. Positive clones express very low but detectable levels of frataxin mRNA These ES cells have been sent to our collaborator Dr. Hélène Puccio at the IGCMB in strasbourg (France) to generate mice carrying the frataxin transgene. These mice will be crossed with Tet-ON transgenics expressing the Tet repressor under the control of a CMV promoter, then with frataxin KO mice. We hope this way to obtain mice expressing a low level of frataxin in the absence of tetracycline, which should be sufficient to allow survival but results in a FA-like disease. Administration of tetracycline should restore frataxin expression and allow studying whether and how the phenotype is reversible. These animals should be able to go through the critical phase of embryonic development when frataxin KO animals die, because the CMV promoter controlling the expression of the Tet repressor, which inhibits frataxin expression, is activated only later, around E8.5.

The second model uses a small inhibitory RNA (siRNA) approach to reduce frataxin expression. It is also used to generate cellular models. Transiently transfected cells show that the approach is effective in reducing frataxin level to <20%, as we could demonstrate by immunoflourescence, flow cytometry and western blot. As in the first model, a Tet-ON inducible system will be utilized for stable transfections and animal models. We have prepared a transgene construct expressing the anti-frataxin siRNA shown to be effective by direct transfection in cell culture under a pol III promoter, followed by a Tet operator sequence. This transgene will be incorporated into a lentivirus construct to infect mouse ES cells, previously transfected with the Tet repressor under the control of a CMV promoter, and generate transgenic animals.

2. Analysis of frataxin function.

We obtained solid evidence of the co-regulation of frataxin expression with other components of the mitochondrial Iron-Sulfur cluster (ISC) assembly machinery (Isu1 and Nsf1) in different cell types and in varying physiological conditions (heme synthesis induction), following up the now published results on frataxin level changes in hemopoiesis (included in Miranda et al., 2004).

We obtained preliminary evidence of a role of frataxin in maintaining mitochondrial DNA conformation and integrity against oxidative stress by comparing mitochondrial DNA conformation in native gels between mice that under-express, normally express or over-express frataxin. This result supports a direct role of frataxin as a mitochondrial iron chaperon, that we will further investigate.

The generation of gene expression profiles in tissues from mice that underexpress frataxin compared to wild-type littermates by DNA chip technology has now been completed. Data analysis is ongoing, it essentially confirms the preliminary data in previous reports that some inflammation, iron homeostasis regulation, oxidative stress response, signal transduction, metabolic, and neuronal development pathway genes are activated. This work is done in collaboration with Dr. Dan Geschwind of the University of California at Los Angeles (UCLA).

Publications with aknowledged FMRE support

- C.J. Miranda, M.M. Santos, K. Ohshima, M. Tessaro, J. Sequeiros, and M. Pandolfo "Frataxin overexpressing mice" FEBS Letters 572:281-288 (2004).

Final Report of the Research Group of

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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 180 G protein-coupled receptor types and subtypes have been functionally characterized to date in mammalian species, and about 100 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. Characterization of orphan receptors

With the aim of identifying the natural ligands of orphan receptors, we have established over the years a collection of cell lines expressing genes encoding putative receptors collected in the databases and the human genome sequencing program. Altogether, about ninety orphan receptors have 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 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. Characterization of two receptors for short chain fatty acids (GPR41 and GPR43)

Short chain fatty acids (SCFAs), including acetate, propionate, and butyrate, are produced at high concentration by bacteria in the gut and subsequently released in the bloodstream. Basal acetate concentrations in the blood (about 100 microm) can further increase to millimolar concentrations following alcohol intake. It was known previously that SCFAs can activate leukocytes, particularly neutrophils. We have identified two previously orphan G protein-coupled receptors, GPR41 and GPR43, as receptors for SCFAs. Propionate was the most potent agonist for both GPR41 and GPR43. Acetate was more selective for GPR43, whereas butyrate and isobutyrate were more active on GPR41. The two receptors were coupled to inositol 1,4,5-trisphosphate formation, intracellular Ca^{2+} release, ERK1/2 activation, and inhibition of cAMP accumulation. They exhibited, however, a differential coupling to G proteins; GPR41 coupled exclusively though the Pertussis toxin-sensitive Gi/o family, whereas GPR43 displayed a dual coupling through Gi/o and Pertussis toxin-insensitive Gq protein families. The broad expression profile of GPR41 in a number of tissues does not allow us to infer clear hypotheses regarding its biological functions. In contrast, the highly selective expression of GPR43 in leukocytes,

particularly polymorphonuclear cells, suggests a role in the recruitment of these cell populations toward sites of bacterial infection. The pharmacology of GPR43 matches indeed the effects of SCFAs on neutrophils, in terms of intracellular Ca^{2+} release and chemotaxis. Such a neutrophil-specific SCFA receptor is potentially involved in the development of a variety of diseases characterized by either excessive or inefficient neutrophil recruitment and activation, such as inflammatory bowel diseases or alcoholism-associated immune depression. GPR43 might therefore constitute a target allowing us to modulate immune responses in these pathological situations (Le Poul et al. 2003). GPR41 and GPR43 constitute, together with the related receptors GPR40 and GPR42 (described in parallel), a new family of receptors for fatty acids of different length, which will likely be involved in a broad range of biological function, including regulation of the immune and endocrine systems.

5. Characterization of chemerin as the natural ligand of the orphan receptor ChemR23

Dendritic cells (DCs) and macrophages are professional antigen-presenting cells (APCs) that play key roles in both innate and adaptive immunity. ChemR23 is an orphan G protein-coupled receptor related to chemokine receptors, which is expressed specifically in these cell types. We have characterized chemerin, a novel chemoattractant protein, which acts through ChemR23 and is abundant in a diverse set of human inflammatory fluids. Chemerin was purified from a human ascitic fluid, on the basis of its biological activity on a ChemR23-expressing cell line, and characterized by mass spectrometry. Its activity was confirmed following its production as recombinant protein in mammalian cell lines. Chemerin is secreted as a precursor of low biological activity, which upon proteolytic cleavage of its COOH-terminal domain, is converted into a potent and highly specific agonist of ChemR23, the chemerin receptor. Activation of chemerin receptor results in intracellular calcium release, inhibition of cAMP accumulation, and phosphorylation of p42-p44 MAP kinases, through the Gi class of heterotrimeric G proteins. Chemerin is structurally and evolutionary related to the cathelicidin precursors (antibacterial peptides), cystatins (cysteine protease inhibitors), and kininogens. Chemerin was shown to promote calcium mobilization and chemotaxis of immature DCs and macrophages in a ChemR23-dependent manner. Therefore, chemerin appears as a potent chemoattractant protein of a novel class, which requires proteolytic activation and is specific for APCs (Wittamer et al. 2003).

We have synthesized a number of peptides derived from the C-terminal domain of human prochemerin and have investigated their functional properties as agonists or antagonists of human chemerinR. We found that the nonapeptide ^{149}YFPGQFAFS^{157} (chemerin-9), corresponding to the C terminus of processed chemerin, retained most of the activity of the full-size protein, with regard to agonism toward the chemerinR. Extension of this peptide at its N terminus did not increase the activity, whereas further truncations rapidly resulted in inactive compounds. The C-terminal end of the peptide appeared crucial for its activity, as addition of a single amino acid or removal of two amino acids modified the potency by four orders of magnitude. Alanine-scanning mutagenesis identified residues Tyr^{149}, Phe^{150}, Gly^{152}, Phe^{154}, and Phe^{156} as the key positions for chemerinR activation. A modified peptide (YHSFFFPGQFAFS) was synthesized and iodinated, and a radioligand binding assay was established. It was found that the ability of the various peptides to activate the chemerin receptor was strictly correlated with their

affinity in the binding assay. These results confirm that a precise C-terminal processing is required for the generation of a chemerinR agonist. The possibility to restrict a medium sized protein to a nonapeptide, while keeping a low nanomolar affinity for its receptor is unusual among G protein-coupled receptors ligands. The identification of these short bioactive peptides will considerably accelerate the pharmacological analysis of chemerin-chemerinR interactions (Wittamer et al. 2004).

6. Further characterization of the $NPFF_1$ and $NPFF_2$ 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 NPFF1 receptors. BIBP3226 and BIBO3304, two selective neuropeptide Y Y_1 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 ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$). Three of these receptors have been described in our laboratory ($P2Y_4$, $P2Y_6$ and $P2Y_{11}$).

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 $P2Y_{11}$ 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. Characterization of the F2L peptide as ligand of the FPRL2 receptor

We have identified the natural ligand of FPRL2, a previously orphan receptor. FPRL2 belongs to a structural family of chemoattractant receptors that contains also the formyl peptide receptor (FPR) and FPR-like receptor (FPRL)1. FPRL2 is characterized by its specific expression on monocytes and DCs. We have isolated from a spleen extract and characterized functionally F2L, a novel chemoattractant peptide acting specifically through FPRL2. F2L is an acetylated aminoterminal peptide derived from the cleavage of the human heme-binding protein, an intracellular tetrapyrolle-binding protein. The peptide binds and activates FPRL2 in the low nanomolar range, which triggers intracellular calcium release, inhibition of cAMP accumulation, and phosphorylation of ERK1/2 MAP kinases through the G_i class of heterotrimeric G proteins. When tested on monocytes and monocyte-derived DCs, F2L promotes calcium mobilization and chemotaxis. Therefore, F2L appears as a new natural chemoattractant peptide for DCs and monocytes, and the first potent and specific agonist of FPRL2 (Migeotte et al. in press). We postulate that F2L is released from cells following their death by apoptosis or necrosis, and this release could play a role in a number of disease situations associated with cell death and immune responses.

9. 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.

In the frame of a collaborative study, we have compared the abilities of orexin-A and orexin-B and variants of orexin-A to activate Ca^{2+} influx and intracellular Ca^{2+} release in human OX_1 and OX_2 receptor- expressing CHO cells. Responses mediated by activation of both receptor subtypes were dependent on extracellular Ca^{2+}, demonstrating activation of Ca^{2+} influx. Truncation of orexin-A reduced much its ability to activate OX_1, and to a lower extent OX_2.

Replacement of amino acids 14 to 26 with alanine was performed in the truncated orexin-A variant (14-33). A strong reduction of potency was produced for both receptors by the replacement of Leu20, Asp25, and His26, suggesting that the determinants involved in the activation of the receptor are conserved between the orexin receptor subtypes (Ammoun et al. 2003).

GPR54 is a receptor for peptides derived from the metastasis suppressor gene KiSS-1. To investigate the intracellular mechanisms involved in the reduction of the metastatic potential of MDA-MB-435S cells expressing GPR54, a time course stimulation by kisspeptin-10 over a period of 25h was performed using cDNA microarrays. Comparison with the bradykinin B_2 receptor revealed a distinct pattern of gene regulation despite a common coupling to the $G_{q/11}$ class of G-proteins. Inhibitors of PLC and PK-C abolished the transcriptional regulation of all tested genes, while an inhibitor of p42/44 affected a subset of genes controlled both by GPR54 and B_2. Among the genes specifically up-regulated by GPR54, we found several proapoptotic genes. Stimulation of GPR54 promoted apoptosis while no significant change was observed after B_2 receptor activation. Our results suggest that the metastasis suppressor properties of GPR54 are mediated in part by cell cycle arrest and induction of apoptosis in malignant cells (Becker et al. 2005).

10. 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).

We also contributed to the structure-function analysis of a natural tachykinin-related peptide (stomoxytachykinin) acting as a ligand of the stable fly (Stomoxys calcitrans) tachykinin receptor, STKR (Poels et al. 2004).

11. Characterization of the knock out model for the PrRP neuropeptide receptor (GPR10).

Prolactin-releasing peptide (PrRP) is a recently described neuropeptide that was isolated from rat brain as the natural ligand of the previously orphan G protein-coupled receptor GPR10/hGR3. PrRP was named following its initial description as a positive regulator of prolactin hormone release by pituitary lactotrophs, and is now described as a regulator of pituitary hormones secretion and feeding behavior. In order to investigate the most relevant and non-redundant physiological roles of the PrRP/GPR10 system in vivo, we generated GPR10-deficient mice. GPR10 knock-out (KO) mice were fertile, transmitted the null allele with the expected Mendelian frequency, and did not display obvious abnormalities. They were tested across a wide range of behavioral and physiological assays. We investigated first the potential dysfunction of the endocrine glands controlled by pituitary hormones. No obvious difference was observed in the histology of pituitary itself, or of mammary gland, ovary, testis, adrenal and thyroid. The anterior pituitary was further evaluated by staining the major cell populations, based on ACTH, GH, LH, FSH and PRL immunoreactivity. No significant differences were observed in terms of distribution, cell number or intensity of staining for corticotrophs, lactotrophs, somatotrophs or gonadotrophs in knock-out versus control animals. No difference was observed for basal serum levels of T4 and testosterone. However, corticosterone levels measured at 8 am were slightly lower in knock-out animals, and the difference became significant for 4 pm values. The release of glucocorticoids was assessed under various acute stress situations. In response to hypoglycemia, hypovolemia and LPS challenge, corticosterone increased strongly in both genotypes, but remained significantly lower in knock-out animals, demonstrating a blunted response of the hypothalamic-pituitary-adrenal (HPA) axis in stress situations. As the involvement of PrRP in the control of CRH release has been suggested, CRH transcripts were measured in hypothalamus by quantitative RT-PCR. No significant differences were found in basal situations, but we observed a decrease in CRH transcript content in GPR10-null mice in the context of insulin-induced hypoglycemia.

Following these observations, we investigated whether the low responsiveness of the HPA axis affects other physiological parameters of KO mice under stress conditions. We tested whether KO mice displayed increased sensitivity to an inflammatory challenge. The animals received an intraperitoneal injection of LPS and galactosamine, at doses described to promote an acute hepatitis of moderate severity in wild-type mice. Plasma TNFa levels were 10-fold higher in KO mice, as compared to controls. Serum levels of alanine aminotransaminase (ALT), a marker of hepatocyte destruction through apoptosis or necrosis, were over a 1000-fold higher in KO mice, suggesting major liver damage, which was confirmed by histological analysis. While limited leukocyte infiltration and cell death were observed in control animals, a massive neutrophil

infiltrate was found in KO mice, together with numerous necrotic and apoptotic cells. The important susceptibility to liver inflammation is attributed to the deficient stress hormone response in these animals.

The physiological consequences of a fasting stress on female mice were investigated. Food withdrawal is indeed known to promote hypothermia and lengthening of the hormonal cycle, through activation of the HPA axis. A smaller corticosterone response was observed in the KO mice, following 48 h of fasting. In basal conditions, KO mice were slightly hypothermic (0.5°C lower than control mice). During the fast, we observed a fall in body temperature for both genotypes, but the fall was less important in KO mice. Following the fasting period, the hormonal cycle of control mice shifted from 5.2 days to 8.2 days. The cycle of KO mice was not modified in basal conditions, but was much less affected by fasting (6.5 days). Once again, knock out animals displayed a relative inability to respond appropriately to stressful conditions.

Glucose level was tested as an additional parameter affected by the activity of the HPA axis. In free feeding conditions, basal glucose levels were moderately decreased in KO mice, while insulin levels were not significantly affected. Following a glucose challenge, a lower peak of plasma glucose was observed, again without significant difference in insulin levels. Following an insulin challenge, glucose levels decreased to much lower levels in KO animals, as compared to controls, demonstrating a higher sensitivity to insulin, as the probable result of decreased corticosteroid tonus.

Catecholamines released under control of the orthosympathic system, contribute also to the restoration of normal glycemia following an hypoglycemic stress. We therefore investigated whether catecholamine production was affected in KO mice. Both in free feeding conditions, and during an hypoglycemic stress following insulin challenge, we observed lower urinary excretion of epinephrine, norepinephrine and catecholamine metabolites in knock out animals. To document further the role of GPR10 in the control of the autonomous nervous system, we investigated heart rate and blood pressure using a non-invasive setting. Basal heart rate was decreased in KO mice as compared to control animals. A tendency toward lower systolic blood pressure was also observed.

Altogether, our observations demonstrate that the PrRP-GPR10 system plays an important role in the control of two complementary hormonal responses to stress situations: the HPA axis and the orthosympathic-catecholamine system. PrRP appears therefore to regulate CRH release in hypothalamic structures, and CRH neurons are well known to be at the cross-road between endocrine and sympathetic activation, playing a central role in the regulation of stress responses. Most of the phenotypic alterations observed in our GPR10 KO model can be linked directly to the lower corticosteroid and catecholamine tonus in basal conditions, and to the blunted response of these systems under a variety of stress conditions (Laurent et al. in preparation).

We have more recently investigated the role of the PrRP-GPR10 system in the control of opiate responses. Opiate drugs are largely used for the treatment of severe pain, but heir use is

limited as a consequence of behavioral adaptation to their prolonged use, which includes the development of tolerance and dependence. Although these adaptive mechanisms have been known for decades, the underlying pathophysiological pathways have not been yet clarified entirely. Adaptive regulation of the opioid receptor signalling pathway at the cellular level has been hypothesized, although more recently, a growing contribution of the plasticity of neuronal networks involving opioidergic neurons has been recognized. In support to this latter hypothesis, a number of neuropeptides, including cholecystokinin, neuropeptide FF and nociceptin/orphanin FQ, have been proposed for their role as modulators of the opioid system. These various peptidergic pathways are collectively designated as an anti-opioid system. Among these peptides, neuropeptide FF. Prolactin releasing peptide (PrRP) is an additional member of the mammalian RF-amide peptide family, and the involvement of PrRP and GPR10 in the processing of nociceptive information was suggested by the presence of this system in hypothalamus, amygdala and brainstem, areas involved in the processing of nociceptive signals.

In a first set of experiments, we explored anxiety-related behaviors and locomotor activity for both genotypes. Both the elevated plus maze and the light and dark box paradigms were used to evaluate the anxiety-related responses. In the elevated plus maze test, the number of entries and the total time spent in the open arms were not significantly different according to genotype. Similarly, in the light and dark box test, no difference was observed for the latency for the first entry or the total time spent in the lit compartment. Evaluated in the rota-rod test, motor coordination was not affected in knockout mice. In actimetry boxes, the spontaneous locomotor activity and the activity following morphine administration (10 mg/kg) were similar for both genotypes.

In a second set of experiments, we measured the spontaneous thermal nociceptive threshold in wild-type and knockout mice using two tests reported with a preferential stimulus integration at spinal (tail-immersion) or supraspinal (hot-plate) levels. In the tail-immersion test, similar nociceptive thresholds were observed for both genotypes. In the hot-plate test, increased jump latency was observed in knockout as compared to wild-type mice. Administration of the opioid receptor antagonist naloxone (1 mg/kg, i.p.) was able to reverse the relative analgesia observed in knockout mice in the hot plate test, without affecting the nociceptive threshold of wild-type animals. Surprisingly, naloxone administration also decreased the nociceptive threshold of knockout mice in the tail-immersion test, below that of treated or untreated controls. The antinociceptive effects of morphine were then investigated in the tail-immersion and hot-plate tests. As expected, morphine induced a dose-dependent antinociception in both genotypes, but this effect was significantly enhanced in knockout mice following administration of the 10 mg/kg (i.p.) dose in the two nociceptive models.

Environmental stress is known to promote potent inhibition of behavioral responses to nociceptive stimuli, and this stress-induced analgesia is mediated by the opioid system. As expected, a forced swim test (5 min at 32°C) induced a marked analgesia in the hot-plate test, which was reversed by naloxone in both genotypes. In agreement with the response observed after morphine administration, the stress-induced analgesia was stronger in knockout mice.

These data suggested an interaction between the opioid system and GPR10. To investigate this interaction further, we evaluated the consequences of the central administration of PrRP (5 nmoles, i.c.v.) on the nociceptive threshold recorded in the tail-immersion test. Intracerebroventricular administration of PrRP promoted an hyperalgesia in wild-type mice, and was able to reverse the analgesia induced by morphine in the tail-immersion test. Administration of PrRP to knockout mice had no effects on nociceptive thresholds, demonstrating that the effects of the peptide are exclusively mediated by the GPR10 receptor.

Repeated opiate administration leads to the development of tolerance to its analgesic effects. Morphine tolerance was evaluated during a five day morphine exposure protocol in the tail-immersion test. As expected, morphine (10 mg/kg, i.p.) induced a strong antinociception on day 1, and from day 2 to 5, the antinociceptive response to morphine decreased slowly, down to basal level in both genotypes. The timing of tolerance development was however delayed significantly in knockout, as compared to wild-type mice.

Chronic morphine exposure produces also a strong physical dependence syndrome as assessed by the characteristic set of behavioral responses to naloxone-evoked withdrawal. This includes a number of somatic signs such as jumps, wet dog shakes, paw tremors and sniffing, and more vegetative signs including ptosis, diarrhoea, mastication, piloerection and tremor. Evidence of physical dependence was observed for both genotypes as evaluated during the observation of abstinence. However, there was a significantly lower incidence of somatic signs of withdrawal in knockout mice than in wild-type mice. No difference between the genotypes was observed for the vegetative symptoms observed. The global withdrawal score confirmed the attenuation of the severity of naloxone-precipitated morphine withdrawal syndrome in knockout mice.

The rewarding properties of opiates were investigated in a conditioned-place preference paradigm. Morphine administration has been reported to display a bell-shaped dose response curve in the conditioned place preference paradigm, with the maximal effect in mice at 6 mg/kg (s.c.) of morphine. In our experiments, the maximal effect of morphine was indeed obtained for the 6 mg/kg dosage in wild-type animals. However, the knockout mice responded maximally to the lower dose of morphine (2 mg/kg, s.c.), whereas a higher dose did not induce any rewarding response. This observation suggests an increased sensitivity to the rewarding properties of morphine in the absence of GPR10.

In order to investigate whether the relative analgesia observed in knockout mice might be related to increased proenkephalin expression, we investigated the distribution of proenkephalin transcripts in the brain of both genotypes by in situ hybridization. No major compensatory changes could however be observed. Using [^{3}H]-DAMGO as radioligand, we also characterized the μ binding sites in a saturation binding assay. Similar K_d and B_{max} values were found on whole brain membranes from the two genotypes. The functional response of the μ receptor was evaluated on brain membranes in a [^{35}S]GTP$_{\gamma}$S binding assay, but the EC_{50} and E_{max} values were also similar for wild-type and knockout mice. From these experiments, it appears therefore that no gross alteration of the opioid system has occurred as a compensatory mechanism in GPR10 knockout mice.

Altogether, our observations establish the specific involvement of GPR10 in the control of pain, and qualify the PrRP-GPR10 system as an anti-opioid system, in addition to the CCK2, ORL1 and NPFF receptors. They suggests that GPR10 might constitute a new pharmacological target for the clinical management of pain, opioid side-effects and the treatment of addictive disorders (Laurent et al. 2005).

12. 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 next investigated the potential contribution of the A_{2a} adenosine receptor by generating CB_1/A_{2a} double deficient mice. The spontaneous locomotor activity was reduced in double knockout as compared to wild-type animals. Emotional-like responses were investigated using the elevated plus-maze and the lit-dark box. Mutant mice exhibited an increased level of anxiety in both behavioural models. The specific involvement of CB_1 and A_{2a} receptors in morphine dependence was evaluated by using A_{2a} knockout mice and CB_1/A_{2a} double mutant mice. The severity of naloxone-precipitated morphine withdrawal syndrome was significantly increased in the absence of A_{2a} adenosine receptors whereas no modifications were observed in the double knockout mice. These results suggest that both receptors participate in the control of emotional behaviour and seem to play an opposite role in the expression of opioid physical dependence (Berrendero et al. 2003).

We also investigated the contribution of A_{2A} adenosine receptors in several behavioural responses of Δ9-tetrahydrocannabinol (THC) related to its addictive properties, including tolerance, physical dependence and motivational effects. For this purpose, we first investigated acute THC responses in mice lacking A_{2A} adenosine receptors. Antinociception, hypolocomotion and hypothermia induced by acute THC administration remained unaffected in mutant mice. Chronic THC treatment developed similar tolerance to these acute effects in wild-type and A_{2A}-knockout mice. However, differences in the body weight pattern were found between genotypes during such chronic treatment. Interestingly, the somatic manifestations of SR141716A-precipitated THC withdrawal were significantly attenuated in mutant mice. The motivational responses of THC were also evaluated by using the place-conditioning paradigm. A significant reduction of THC-induced rewarding and aversive effects was found in mice lacking A_{2A} adenosine receptors in comparison with wild-type littermates. Binding studies revealed that these behavioural changes were not associated with any modification in the distribution and/or functional activity of CB_1 receptors in knockout mice. These results indicate a specific involvement of A_{2A} receptors in the addictive-related properties of cannabinoids (Soria et al. 2004).

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).

The role of the adenosine A_{2a} receptor in the hypnotic effects of ethanol was assessed in another study. The duration of the loss of righting reflex following acute ethanol administration was shorter for A_{2a} receptor-deficient mice than for wild-type mice, whereas the fall in body temperature was not different between the two genotypes. In contrast, the duration of the loss of righting reflex was increased in KO mice versus controls after administration of pentobarbital. Dipyridamole, an inhibitor of adenosine uptake, increased the sleep time observed following administration of ethanol in control but not in KO mice. SCH58261, a selective A_{2a} receptor antagonist, unlike DPCPX, a selective A_1 receptor antagonist, shortened the duration of the loss of righting reflex induced by ethanol, thus mimicking the lack of receptor in deficient mice. Finally, the non-selective adenosine receptor antagonist caffeine reduced ethanol-induced hypnotic effects. These results indicate that the activation of A_{2a} receptors that follows an increase in extracellular adenosine levels caused by the administration of high doses of ethanol plays a role in its hypnotic effects (El Yacoubi et al. 2003).

A_{2a} receptor knockout mice were shown to be more anxious and aggressive, and exhibit reduced exploratory activity than their wild-type littermates. Because α-MSH influences anxiety, aggressiveness and motor activity, the effect of $A_{2a}R$ gene disruption on alpha-MSH content in brain regions was investigated, as well as pro-opiomelanocortin (POMC) expression in the hypothalamus and pituitary. A significant increase in alpha-MSH content was observed in the amygdala and cerebral cortex, two regions that are innervated by POMC terminals, but not in hypothalamus and medulla oblongata. POMC mRNA levels were not affected in the arcuate nucleus of the hypothalamus. A substantial increase in POMC mRNA and adrenocorticotropin hormone concentrations was observed in the anterior lobe of the pituitary, and plasma corticosterone concentration was significantly higher in knockout mice, revealing hyperactivity of their pituitary-adrenocortical axis. Together, these results suggest that adenosine, acting through A_{2a} receptors, may modulate the release of alpha-MSH in the cerebral cortex and amygdala. The data also indicate that A_{2a} receptors are involved in the control of POMC gene expression and biosynthesis of POMC-derived peptides in pituitary corticotrophs (Jegou et al. 2003).

Adenosine is considered as an extracellular mediator that protects cells from various types of metabolic injuries, including hypoxic ischemia brain damage. The role of the A_{2a} receptor in this process was investigated using 7-day-old A_{2a} knockout $A_{2a}R^{-/-}$ mice in a model of hypoxic ischemia, induced by exposure to 8% oxygen after occlusion of the left common carotid artery. Reduction in cortical cerebral blood flow during hypoxic ischemia and rectal temperature did not differ between wild-type and knockout mice. The resulting lesion was evaluated by histopathological scoring after 5 days, 3 weeks and 3 months. Brain injury was aggravated in knockout mice as compared with wild-type mice. Knockout mice also displayed increased forward locomotion and impaired rotarod performance in adulthood compared with control mice, whereas beam-walking performance was similarly defective in both groups. These results suggest that, in contrast to the situation in adult animals, $A_{2a}R$ plays an important protective role in neonatal hypoxic ischemia brain injury (Aden et al. 2003). Other experiments using the knockout model have

shown that coronary vascular responses to endogenous adenosine are mediated by activation of both A_{2a} and A_{2b} receptors in isolated mouse hearts (Taludker et al. 2003).

Previous studies have demonstrated a decrease in [^{3}H]nitrobenzylthioinosine binding sites in the brainstem of adenosine A_{2a} receptor knockout mice, particularly in the brain nuclei involved in central control of cardiovascular function. This decrease, shown using autoradiography, was now correlated with a functional change using a previously described method of [^{3}H]adenosine uptake in a membrane preparation from the brainstem of wildtype CD1 and homozygous mutant mice lacking the adenosine A_{2a} receptor. A statistically significant decrease was shown in the mean V_{MAX} value obtained from homozygous mutant preparations compared to that obtained from wildtype controls. Competition studies using nucleoside uptake inhibitors showed a statistically significant increase in the log IC_{50} values for dipyridamole and dilazep in the preparations using homozygous mutant tissue. These results indicate that components of purinergic neurotransmission system have apparently adjusted in compensation for the lack of the A_{2a} receptor (Snell et al. 2004).

Caffeine has biphasic effects on locomotion, and blockade of the adenosine A_{2A} receptor ($A_{2A}R$) is necessary for the stimulatory effect of low doses of caffeine, but not for the locomotor depressant effect observed at high doses. The role of the adenosine A_1 receptor (A_1R) in mediating the locomotor effects of increasing doses of caffeine was studied by using wild-type mice, mice heterozygous for, and mice lacking the adenosine A_1 receptor (A_1R^{KO}). Caffeine had the typical biphasic dose-effect relationship in all three genotypes, but the stimulatory action of caffeine was facilitated in the A_1R^{KO} mice. In order to investigate the interaction between blockade of A_1Rs and A_{2A}Rs, mice lacking both receptors ($A_1R^{KO}/A_{2A}R^{KO}$) were tested. Regardless of A_1R genotype, animals lacking A_{2A}R were not stimulated by caffeine, whereas animals heterozygous for A_{2A}R were. As expected, the A_1R is not crucial for the stimulatory effect of caffeine, but seems to modulate the effect of caffeine exerted via A_{2A}R blockade. Furthermore, these results suggest that the inhibitory effect of high doses of caffeine is due neither to blockade of the A_1R, nor of the A_{2A}R, and an effect independent of these adenosine receptors is likely (Halldner et al. 2004).

13. Characterization of a mouse knock-out model for the central cannabinoid receptor CB_1.

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). The role of cannabinoid CB_1 receptors in the action of anxiolytics was examined. Deletion of CB_1 receptors resulted in increased anxiety-like behaviours in light/dark box, elevated plus maze and social interaction tests. Mutant mice presented basal low corticosterone concentrations and low proopiomelanocortin gene expression in the anterior lobe of the pituitary gland compared to wild-type mice. Ten minutes of restraint stress resulted in a twofold increase in corticosterone concentrations in the plasma of mutant mice, compared to wild-type mice. Bromazepam (50 or 100 microg/kg) markedly increased the time spent in light area in wild-type animals, though both doses were without effect in mutant mice. Administration of buspirone (1 or 2 mg/kg) produced anxiolytic effects in wild-type mice. In contrast, only the highest dose of buspirone had anxiolytic results in mutant mice. Our findings reveal that CB_1 receptors are involved in the regulation of emotional responses, and play a pivotal role in the action mechanism of anxiolytics. They suggest that alterations in the functional activity of the CB_1 receptor may be related to the emergence of anxiety disorders, and may affect treatment with anxiolytics (Uriguen et al. 2004).

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).

The effects of the endogenous cannabinoid anandamide, on the activity of the hypothalamo-pituitary-adrenal (HPA) axis was investigated. Anandamide increased plasma corticotropin (ACTH) and corticosterone concentrations in both wild-type and CB_1 receptor KO mice. Selective antagonists of the CB_1 and vanilloid VR1 receptors did not prevent the effects of anandamide. Using Fos protein immunohistochemistry, an activation of the parvocellular part of the hypothalamic paraventricular nucleus (PVN) was observed 45 min after anandamide injection in both genotypes. These results support the view that activation of the HPA axis produced by anandamide occurs via a currently unknown (CB_x) cannabinoid receptor present in PVN (Wenger et al. 2003). In a second study, basal and novelty stress-induced plasma levels of adrenocorticotropin (ACTH) and corticosterone were higher in CB_1-KO than in WT mice. The involvement of the pituitary in the hormonal effects of CB_1 gene disruption was investigated by studying the in vitro release of ACTH from anterior pituitary fragments using a perifusion system. Both the basal and corticotropin releasing hormone (CRH)-induced ACTH secretion were similar in CB_1-KO and WT mice. The synthetic glucocorticoid, dexamethasone suppressed the CRH-induced ACTH secretion in both genotypes; thus, the negative feedback of ACTH secretion was not affected by CB_1 gene disruption. The cannabinoid agonist, WIN 55,212-2 had no effects on basal and CRH-stimulated ACTH secretion by anterior pituitary slices. In our

hands, the disruption of the CB_1 gene lead to HPA axis hyperactivity, but the pituitary seems not to be involved in this effect. These data are consistent with the assumption that endogenous cannabinoids inhibit the HPA-axis via centrally located CB_1 receptors (Barna et al. 2004). Another study investigating the inhibition of cannabinoids on depolarization-evoked release of [^{3}H]glutamate from hippocampal synaptosomes suggested also an independance from the CB1 receptor (Kofalvi et al. 2003).

Recent data suggest that the endocannabinoid system may play a key role in the reinforcing effects of ethanol. The CB_1-KO mice were used to analyse this further. Disruption of CB1 receptors decreased both ethanol consumption and preference. This decreased ethanol self-administration was associated with increased sensitivity to the acute intoxicating effects of ethanol. Mutant mice were more sensitive to the hypothermic and sedative/hypnotic effects of acute ethanol administration (1.5-4.0 g/kg), although plasma ethanol concentrations did not differ from those of controls. Moreover, wild-type mice exhibited normal locomotor activation caused by 1.0-2.5 g/kg injection of ethanol, whereas mutant mice displayed sedation in response to the injection of the same ethanol doses. The severity of alcohol withdrawal-induced convulsions was also increased in CB1-KO mice. Our results suggest that CB1 receptors participate in the regulation of ethanol drinking and demonstrate that their disruption lead to increased ethanol sensitivity and withdrawal severity (Naasila et al. 2004).

14. 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.

CCR5 was shown to homodimerize but also to heterodimerize with CCR2b, a closely related receptor. We have analyzed the functional consequences of this dimerization process, in terms of ligand binding, stimulation of intracellular cascades and internalization. BRET and co-immunoprecipitation assays demonstrated that CCR5 and CCR2b heterodimerize with the same efficiency as they homodimerize. In contrast to what has been reported earlier, no cooperative signaling was observed following co-stimulation of the two receptors by their respective ligands. However, we observed that CCR5-specific ligands which are unable to compete for MCP-1 binding on cells expressing CCR2b alone, efficiently prevented MCP-1 binding when CCR5 and CCR2b were co-expressed. The extent of this cross-competition was correlated with the amount of CCR5 expressed in cells, as determined by FACS analysis. Similar observations were made for the CCR2b-selective ligand MCP-1, that competed efficiently for MIP-1β binding on cells expressing both receptors. Internalization assays did not allow us to demonstrate co-internalization of the receptors in response to agonist stimulation. Taken together, our observations suggest that CCR5 and CCR2b form homo- and heterodimers with similar efficiencies, and that a receptor dimer can only bind a single chemokine (El Asmar et al. 2004).

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-1a 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). We have also contributed to a study showing that CCR5 signaling is not required for efficient infection of primary T lymphocytes and macrophages (Amara et al. 2003).

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Mechanisms of long-term potentiation in normal and pathological memory

Our laboratory is priorily interested in the mechanisms of memory and of its pathology (in Alzheimer's disease, for instance). Nowadays, there is a general consensus that encoding of memories relies on modifications of the strength of synaptic connections. In our laboratory, the mechanisms of these modifications are studied in vitro on two well known models: (1) the long-term potentiation induced in an hippocampal slice by high frequency stimulation of a bundle of axons and (2) synaptic plasticity triggered by diverse agents on a culture of hippocampal neurons.

1. LTP on slices from transgenic mice generated to explore memory

From January to September 2003, Laurence RIS worked in the laboratory of Learning and Memory (headed by Prof. GIESE) at the University College of London. This laboratory of molecular biology is specialized in creating mutations (knock out, overexpression, mutation) in genes suspected to play a role in the memory of the mouse. We decided to combine their expertise in that field with ours in the electrophysiological exploration of long-term potentiation in hippocampal slices maintained artificially alive in vitro. One work was completed in 2004 and was submitted for publication in January 2005. It shows that the gender of the animal has a great influence on the role played by the α- and β- isoforms of calcium/calmodulin kinase kinase (CaMKKα and CaMKKβ) in memory and LTP (Mizuno et al., submitted).

2. LTP in slices from transgenic mice in relation with Alzheimer's disease

For several years we have been collaborating with the laboratory of Prof. VAN LEUVEN (KUL) which has generated different strains of transgenic mice in relation with Alzheimer's disease (AD). 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).

Recently, in collaboration with Prof. FAHRENHOLZ in Germany, we demonstrated that overexpression of α secretase (ADAM 10) corrected the deficit in the LTP observed in our transgenic model of AD (Journal of Clinical Investigation, 2004).

On the other end, it has been observed that p25 protein was overexpressed in the brains of patients with AD. Overexpression of high level of p25 in transgenic mice induces degeneration of the brain similar to that observed in AD. The laboratory of Prof. GIESE has expressed p25 at a low level. Using these transgenic mice, we found that this type of expression had surprising consequences: certain types of memory are improved whereas LTP is altered in a sex-linked way (Ris et al., submitted).

3. L-LTP in slices from normal mice

There are at least two types of LTP. One lasts between 1 and 3 h (E-LTP, early LTP), the other last longer than 4 h (L-LTP, late LTP). The mechanisms of the first are well known. The mechanisms of the latter are not. During last year, we explored the role of phosphorylation in L-LTP. L-LTP was induced by a perfusion of forskolin-IBMX and small samples of the CA1 region of the treated slices were submitted to a 2-D electrophoresis.

During our investigations, we also found that synaptic strength was influenced not only by the activity of the neurons (induced by electrical stimulation) but also by the past history of the slices.

4. Synaptic plasticity in neuronal cultures

During last year, we also submitted cultures of hippocampal neurons to agents like forskolin, a well known activator of adenylate cyclase. The efficacity of the synapses was assessed by analysing the amplitude and frequency of the miniature excitatory post-synaptic currents (mEPSC) recorded in a single neuron using the patch-clamp whole-cell technique. The mechanisms of the discovered phenomenon are in progress.

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I. Physiology and physiopathology of the basal ganglia system

Gene targeting of the striatal neuronal subpopulations to investigate the roles of indirect and direct pathways of basal ganglia

Our aim was to generate transgenic mice allowing the study of the specific roles of striatopallidal or striatonigral neurons. We have obtained mice strains expressing the CRE recombinase under the control of the A_{2A} receptor promotor inserted in a BAC (bacterial artificial chromosome) to direct gene expression specifically in striatopallidal neurons. These lines have been crossed with a reporter strain (Rosa26-LacZ) in order to determine whether they selectively expressed CRE in these striatopallidal neurons. Co-localisation experiments using anti-enkephalin antibody (Enk) and LacZ activity detection showed that >80% of the LacZ neurons are also Enk+. About 15% of LacZ+ neurones are Enk-, but this could be due to low detection of ENK at the somatic level. Retrograde labelling of striatonigral neurons combined with the immunodetection of β-galactosidase showed that striatonigral are LacZ-negative. Therefore, altogether, these results demonstrated that we have generated strains of transgenic mice with a specific CRE expression in the striatopallidal neurons. Having these neuron-specific transgenic lines, we have crossed them with strains of "floxed" mice allowing the selective inactivation of genes in this population of neurons. The NMDA receptors seem to play a key role in reward and drug addiction (ventral striatum) and in motor learning (dorsal striatum). Moreover, NMDA receptors are supposed to be involved in synaptic plasticity at the cortico-striatal and cortico-accumbal synapses (LTP and LTD). Synaptic plasticity in these areas is strongly suggested to be the basis for motor learning and addiction. An important question is therefore the specific role of this receptor in each subpopulation. The NR1 floxed mice has been previously generated and published and have been obtained from Prof. Tonegawa and collaborators (MIT, Boston, USA) allowing a conditional inactivation of NR1 by the CRE recombinase. A colony of these mice has been established and the resulting mice were then crossed with our A2A-CRE mice. First series of animals to be tested will be obtained in a near future.

Neuronal death and neuroprotection in models of Huntington's disease

We previously developed an experimental model of Huntington's disease in rat or mouse by using subchronic injection of 3-nitropropionic acid (3NP). We previously demonstrated the high reproducibility of this model in terms of lesion and evaluated the potential neuropotective effects of adenosine A_{2A} or A_1 receptors ligands. These results have been reviewed in comparison with results obtained in other models of Huntington's disease or neurodegenerative diseases (7). In this model, we have also characterized the loss of dopaminergic afferents in the striatum and suggested that this is a secondary consequence of the striatal neuronal death (1). We described also the induction of expression of a protein, Alix, which is specifically involved in apoptosis processes (2). Minocycline has been a proposed as a neuroprotective agent in several models of neurodegenerative diseases. We evaluated its neurprotective potency in different models of Huntington's disease and demonstrated that it could slow down the development of inflammation and of caspases-induced neuronal death but not the development of calpain -dependant neuronal (10).

In order to identify mechanisms leading to the selective striatal neuronal death in Huntington's disease, we have comparatively characterized processes of 3NP-induced neuronal death on striatal and cortical neurons in primary cultures (6). Although, 3NP-induced degeneration is similar in both neuronal populations, mechanisms are radically distinct. In striatal neurons, 3NP produces a translocation of Bad, Bax, cytochrome c and Smac whilst this is not observed at all in cortical neurons. The death of striatal neurons is preceded by calpain activation and is blocked by an inhibitor of calpain. This is not observed in cortical neurons. In both neuronal types, neuronal death is independent on the activation of caspases -9 and -3. These results demonstrated that in case of mitochondrial inhibition, striatal and cortical neurons died through different pathways (6).

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

We had characterized cellular mechanisms leading to the alterations observed in calretinin-deficient mice through an approach combining electrophysiology in vitro (patch clamp in the perforated patch configuration) of granular cells of the cerebellum and computer modeling. We demonstrated that the absence of calcium buffering modifies the intrinsic excitability through a modification of the response of calcium-activated potassium channels. We extended these data by using different mathematical models and reviewed these results in a general perspective (5).

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. In vitro experiments using patch clamp technique in these strains of mice demonstrated that the rescue of calretinin expression in granular cells restores a normal intrinsic excitability of these neurons. Moreover, in vivo electrophysiology experiments demonstrated that the rescue of calretinin in granule cells dose-dependently restores a normal firing behavior of Purkinje cells recorded in alert mice. Finally, behavioural analysis of the motor coordination also showed that the rescue expression of calretinin only in cerebellar granule cells is sufficient to restore a normal phenotype for all parameters (12).

We described a fast (160 Hz) local field potential oscillation recorded in vivo through extracellular recordings in the cerebellar cortex of mice deficient in calcium binding proteins (3,4). We suggested that this oscillation was generated by Purkinje cells whose behavior became rythmic and synchronous in these mice. We have pharmacologically identified NMDA and GABAa receptors as well as gap junctions as requested to generate this oscillation. This

constitutes the first description of a fast oscillation in the cerebellum whereas such electrophysiological behaviors have been reported in other brain areas such as cerebral cortex, hippocampus and thalamus where it is proposed that they play important functional roles (3,4).

We have characterized in vivo at the Purkinje cell level, the influence of the simple spike discharge pattern on the form of the complex spike. This allowed to describe a new form of short term plasticity (9).

III. Molecular characterization of the gastrointestinal pacemaker mechanism.

Contribution of the SK3-expressing fibroblast-like cells (FLC)

FLC expressing the "calcium activated potassium channel" SK3 channel are a new functional type of cells that we have previously identify in the smooth muscle of the gastrointestinal tract, besides interstitial cells of Cajal (ICC). We hypothesised that these cells may play a role in the excitability of gut smooth muscle and in its response to impulses from the enteric nervous system, therefore influencing gut motility. To elucidate the function of these cells, we studied transgenic mice SK3 tTA, carriers of the conditionnal inactivation of SK3, (Prof. Adelman, Portland). The transgenic SK3-tTA model allows to compare wild-type, SK3-overexpressing and SK3-knock-out mice in a same background. We have analyzed in vivo the gastric emptying and the gut transit. In vitro experiments in organ bath allowed to record the rest potential, small depolarization waves and inhibitory response to the electrical stimulation of the enteric nervous system (purinergic component sensitive to apamine). Our results demonstrate a significant difference in the time of gut transit and in the inhibitory response to the electrical stimulation without alteration in small depolarization waves.

Identification of genes involved in the pacemaker function of ICC

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 mouse jejunum but absent in the jejunum of two independent strains of mice deficient in ICC (W^{lacZ}/W^{v} and Sl/Sld) mice. Candidate genes picked up in 2 independent experiments have been evaluated by quantitative PCR, Northern blotting and immunohistochemistry. This approach allowed us to identify and characterize four different genes whose one is KIT, validating therefore the method, and another codes for the *bumetanide sensitive sodium potassium 2 chloride co-transporter* (NKCC1). We confirmed the selective expression of this transporter in ICCs by double immunoflurorescence and confocal microscopy. Functional experiments in vitro using pharmacological tools as bumetamide and transgenic NKCC1 knock-out mice (Prof. E. Delpire, Vanderbilt University, Tennessee) showed that NKCC1 play a modulatory role in the rhythmic electrical activity of the gut smooth muscle. A manuscript describing these results is in preparation.

Gut motility in human diseases and animal models

We have contributed to the description of mutations in nNOS isoforms in Hirschsprung's disease (8) and characterized a new spontaneous mutation in mice (Pretty mice, Pm). These latter mice presented an increased number of ICCs leading to a increased activity of the intestinal pacemaker and hence to an increased gut motility. (11).

PUBLICATIONS 2004

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Understanding neurological diseases: a molecular genetic approach

1. 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*) (Rademakers et al. Human Mutation 24:277-295,2004). 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 A*ß* 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 patients, including autosomal dominant patients, 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

Genome-wide linkage analyses

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, no mutation was identified in the known AD genes APP, PSEN1 and PSEN2.

We performed a genome-wide scan in the two most informative Dutch families 1083 and 1270. In family 1083, a 4-generation pedigree with autosomal dominant early-onset dementia (mean onset age: 64.9 years, range 53 - 79 years), we obtained conclusive linkage with chromosome 17q21 markers with a maximum multi-point LOD score of 5.51 at D17S951 and identified a candidate region of 4.8 cM between D17S1787 and D17S958 containing MAPT. Recent clinical and neuropathological follow-up of the family showed that the phenotype most closely resembled frontotemporal dementia (FTDU) characterized by dense ubiquitin-positive neuronal inclusions that were tau negative. Extensive mutation analysis of MAPT identified 38 sequence variations in exons, introns, untranslated regions and the 5' regulatory sequence, however none were comprised within the disease haplotype. Although our findings do not entirely exclude a mutation in a yet unanalyzed region of MAPT, the apparent absence of MAPT mutations combined with the lack of tau pathology is highly suggestive for another defective gene at 17q21 responsible for FTD in this family. We hypothesized that the dementia in family 1083 could result from mutations in the gene encoding presenilin homologue 2 (PSH2), located 50 kb upstream of MAPT. We performed a mutation analysis of the coding and regulatory regions of PSH2, and identified 5 novel and 10 known single nucleotide polymorphisms (SNPs) (Rademakers et al. Neurogenetics 5: 79-80, 2004). Segregation analysis in the family indicated that only 1 novel SNP g.1626G>A was contained in the disease haplotype leading to a silent mutation at codon S542. We identified the A allele in 27 of 190 control chromosomes (14%). Therefore, we conclude that another defective gene at 17q21 should be considered for this subtype of FTD.

In family 1270, a 3-generation Dutch family with mean onset of disease of 66.8 ± 7.4 years (range 47 - 77), we obtained a genomewide significant 2-point linkage of AD with D7S798 at 7q36 (Z = 3.39, ? = 0.0). Genetic finemapping defined a candidate region of 19.7 cM with a maximum multi-point LOD score of 3.47 at D7S1807. The assembled and annotated genomic sequence indicated a physical size of 5.44 Mb and 29 genes. Mutation analysis of all coding exons identified a synonymous SNP located in the PAX transactivation domain-interaction protein (PTIP) gene that cosegregated with AD, and was absent from 320 control individuals. RT-PCR and Northern blot analysis showed that PTIP is expressed in brain regions affected in AD. Alignment of PTIP cDNA with gDNA indicated that the silent g.38030G>C at codon Ala626 is located in exon 10 of PTIP at 10 bp from the intron 10 splice donor site. PTIP, contains BRCT domains known to function as protein-protein interaction modules enabling the assembly of multiprotein complexes active in DNA recombination or repair, in cell cycle control, as well as in gene activation.

Recruitment of new families - mutation studies

Additional informative families are also being recruited through a proband with early-onset dementia and absence of mutations in the known AD genes. Probands are identified in the DNA diagnostic unit and a prospective study of dementia performed in the Middelheim Memory Clinic, Antwerp (Engelborghs et al. J Neurol Neurosurg Psychiat 74:1148-1151, 2003). In screening the known AD genes for mutations, several mutations are found (http://molgen-www.uia.ac.be/ADMutations/).

In a 6-generation Belgian family with autosomal dominant early-onset dementia, we identified a R406W mutation in tau. In this family, AD rather than FTD, was the main clinical diagnosis. R406W was previously identified in 2 other extended dementia families originating from Western Europe. Haplotype analysis in a 180 kb region including MAPT did not support a common founder for R406W in the 3 families (Rademakers et al., Hum Mutation 22:409-412, 2003).

In a German patient with presenile AD, we identified a novel missense mutation (V715A) near the γ-secretase cleavage site of the amyloid precursor protein gene (APP). The patient was diagnosed with probable AD according to the NINCDS-ADRDA criteria, had an onset age of 48 years and a family history compatible with autosomal dominant AD. Similar to other γ-secretase site mutations in APP, this mutation leads to a 4.1 times increased $Aß_{42}$ to $Aß_{40}$ ratio in HEK293 cells (Cruts et al., J Neurol 250: 1374 -1375, 2003).

Familial forms of FTD with tauopathy are mostly caused by MAPT mutations. However, rare forms of familial tauopathy without MAPT mutations have been reported, suggesting other tauopathy-related genetic defects. Interestingly, 2 PSEN1 mutations (Leu113Pro and insArg352) have been recently associated with familial FTD albeit without neuropathological confirmation. We identified a novel PSEN1 mutation in a patient with Pick-type tauopathy in the absence of extracellular Aβ deposits. The mutation is predicted to substitute Gly -> Val at position 183 (Gly183Val) and to affect the splice signal at the junction of the 6th exon and intron. Further clinical-genetic investigation revealed a positive family history of FTD-like dementia and suggested that Gly183Val is associated with a phenotypically heterogeneous neurodegenerative disorder. Our results establish PSEN1 as a candidate gene for Pick-type tauopathy without MAPT mutations (Dermaut et al. Annals of Neurology 55: 617-626, 2004).

Despite advances in elucidating the genetic epidemiology of AD and FTD, the etiology for most patients with dementia remains unclear. We examined the genetic epidemiology of dementia in a recent genetically isolated Dutch population founded around 1750. The series of 191 patients ascertained comprised 122 probable AD patients with late onset and 17 with early onset, and 22 with possible AD. It further included 10 patients with vascular dementia, nine with DLB and six with FTD. All patients, except those with vascular dementia, were more closely related than healthy individuals from the same area. Clustering was strongest for patients with early-onset AD or DLB. Although 14% of late-onset AD patients had evidence of autosomal dominant disease, consanguinity was found in three late-onset AD patients, suggesting a recessive or polygenic model underlying the trait. We found no clustering of vascular dementia, implying a difference in genetic risk for late-onset AD and vascular

dementia. Mutations in known genes could not explain the occurrence of dementia, but the population attributable proportion of APOE ε4 was high (45%) due to a high frequency of APOE ε4 carriers. Earlier identified regions on chromosomes 10 and 12, nor the effect of the alpha-2-macroglobulin (A2M) I/D polymorphism on AD could be confirmed in our study. We did find evidence for association between the A2M D-allele and DLB. Our data showed a strong familial clustering of various forms of dementia in this isolated Dutch population. A high percentage of late-onset AD could be explained by APOE ε4, but 55% of its origin is still unknown (Sleegers et al. Brain 127(7): 1641-1649, 2004).

Identification of susceptibility genes for dementia

We have large population-based and hospital-based patient/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.

Nicastrin haplotype

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. These results further emphasize the importance of γ secretase dysfunction in the etiology of familial presenile AD.

In AD the most characteristic neuropathological features are Aß accumulations in neuritic plaques in AD brain. Nicastrin (NCSTN), a type 1 transmembrane glycoprotein, was shown to modulate Psens and Aß production. Recently we provided evidence that genetic variations in the NCSTN gene modified the risk for familial early-onset AD in a Dutch population-based sample. Risk was highest in the cases that did not carry an APOE ε4 allele. In the present study, we evaluated association of NCSTN polymorphisms to the occurrence of AD in 133 patients and 189 matched controls ascertained in the eastern Finnish population. No statistically significant differences were observed with the exception of a positive trend in the AD patients without APOE ε4 allele (Helisalmi et al. Neurology 63(1): 173-175, 2004).

Prion protein

There is an increasing interest in the role of the M129V polymorphism in the prion protein gene (*PRNP*), a risk factor for Creutzfeldt Jacob Disease (CJD), in AD. We analyzed the M129V in a Dutch population-based early-onset AD sample and observed a significant association between

early-onset AD and homozygosity of M129V (odds ratio [OR], 1.9; 95% confidence interval [CI], 1.1-3.3; p = 0.02) with the highest risk for V homozygotes (OR, 3.2; 95% CI, 1.4-7.1; p < 0.01). In patients with a positive family history, these risks increased to 2.6 (95% CI, 1.3-5.3; p < 0.01) and 3.5 (95% CI, 1.3-9.3; p = 0.01), respectively (Dermaut et al. Annals of Neurology 53(3): 409-412, 2003).

Functional characterization of dementia genes

We have previously shown that CC homozygosity at the -22C>T promoter polymorphism in *PSEN1* is associated with increased risk for AD (Theuns et al. Human Molecular Genetics 9(3): 325-331, 2000). Also, studies in AD brains suggested that CC homozygosity increased the risk for AD by increasing the Aß load. We characterized the *PSEN1* promoter by deletion mapping, and analyzed the effect of the -22C and -22T alleles on the transcriptional activity of *PSEN1* in a transient transfection system. We showed a neuron-specific 2-fold decrease in promoter activity for the -22C risk allele, which in homozygous individuals would lead to a critical decrease in *PSEN1* expression. The deletion mapping suggested that the 13 bp region (33/ 20) spanning the -22C>T polymorphism harbors a binding site for a negative regulatory factor. This factor has a higher affinity for the -22C risk allele and is strongly dependent on downstream sequences for cell type specific expression differences. Together, these studies provide evidence that the increased risk for AD associated with *PSEN1* may result from genetic variations in the regulatory region, leading to altered expression levels of *PSEN1* in neurons (Theuns et al. Human Molecular Genetics 12(8): 869-877, 2003).

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2. PERIPHERAL NEUROPATHIES

We aimed 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 40 loci and identified 32 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 was our priority to make optimal use of these opportunities and to force breakthroughs 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 a summary of our data on our mutation screening in known and novel genes for IPN. These mutations are regularly updated in our mutation database (Inherited Peripheral Neuropathies Mutation Database, IPNMD at: http://molgen-www.uia.ac.be/CMTMutations/).

Genotype/phenotype correlations

Charcot-Marie-Tooth neuropathy type 1C (CMT1C) is an autosomal dominant demyelinating peripheral neuropathy caused by missense mutations in the small integral membrane protein of lysosome/late endosome (SIMPLE) gene. To investigate the prevalence of *SIMPLE* mutations, we contributed to the screening a cohort of 152 probands with various types of demyelinating or axonal, and pure motor or sensory inherited neuropathies. *SIMPLE* mutations were found only in CMT1 patients. Haplotype analysis of short tandem repeats (STRs) and intragenic single nucleotide polymorphisms (SNPs) linked to the gene, demonstrated that families with the same mutation are unlikely to be related. The clustering of the mutations within 5 amino acids suggests that this domain may be critical to peripheral nerve myelination. Electrophysiological studies showed that CMT1C patients from six pedigrees had reduced nerve conduction velocities (NCVs). Two patients had temporal dispersion of nerve conduction and irregularity of conduction slowing, which is unusual for CMT1 patients (Bennett et al. Annals of Neurology 55: 713-720, 2004).

Mutations in *NEFL* were reported as a cause for autosomal dominant CMT2E linked to chromosome 8p21. In order to investigate the frequency and phenotypic consequences of NEFL mutations, we screened 323 patients with CMT or related peripheral neuropathies. We detected 6 disease-associated missense mutations and one 3-bp in-frame deletion clustered in functionally defined domains of the NEFL protein. Patients have an early onset and often a severe clinical phenotype, resembling the Dejerine Sottas (DSS) phenotype. Electrophysiological examination shows moderately to severely slowed NCVs. We reported the first nerve biopsy of a CMT patient with a de novo missense mutation in *NEFL*, and found an axonal pathology with axonal regeneration clusters and onion bulb formations (Jordanova et al. Brain 126: 590-597, 2003).

Autosomal recessive demyelinating Charcot-Marie-Tooth disease (CMT4), DSS and congenital hypomyelinating neuropathy (CHN) are variants of hereditary demyelinating neuropathy of infancy, a genetically heterogeneous group of disorders. To explore the spectrum of early-onset demyelinating neuropathies further, we studied the clinicopathological and genetic aspects of 20 patients born to unaffected parents. In 19 families out of 20, consanguinity between the parents or presence of an affected sib suggested autosomal recessive transmission. Screening of various genes known to be involved in CMT4 revealed 6 mutations of which five are novel. Four of these novel mutations occurred in the homozygous state and include: one in ganglioside-induced differentiation-associated protein 1 gene (*GDAP1*), one in myotubularin related protein 2 (*MTMR2*), one in periaxin (*PRX*) and one in KIAA1985. One patient was heterozygous for a novel MTMR2 mutation and still another was homozygous for the founder mutation, R148X, in NDRG1. All patients tested negative for mutations in EGR2. Histopathological examination of nerve biopsy specimens showed a severe, chronic demyelinating neuropathy, with onion bulb formation, extensive demyelination of isolated fibres and axon loss. We did not discern a specific pattern of histopathology that could be correlated to mutations in a particular gene (Parman et al. Brain 27(Pt 11): 2540-2550, 2004).

Mutations in *PRX* on chromosome 19q13 were reported in DSS and CMT4F. PRX is a novel cytoskeleton 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 and frameshift mutation in a Turkish and Belgian family respectively (Takashima et al. Annals of Neurology 51: 709-715, 2002).

We screened *MTMR2* gene, associated with recessive CMT4B1 with myelin outfoldings linked to chromosome 11q22, and observed a homozygous missense mutation in a consanguineous Turkish CMT family (Nelis et al. Neuromuscular Disorders 12(9): 869-873, 2002).

Mutations in *GDAP1* were recently shown to be responsible for autosomal recessive demyelinating CMT as well as autosomal recessive axonal CMT with vocal cord paralysis on chromosome 8q21.1. We identified in *GDAP1* frameshifts and missense mutations in the homozygous or compound heterozygous state. *GDAP1* mutations seem to be a frequent cause of autosomal recessive CMT, and result in an early onset severe clinical phenotype. The range of NCV was variable: some patients had normal or near normal NCVs suggesting an axonal neuropathy, while others had severely slowed NCVs compatible with demyelination. The neuropathological findings were equally variable and showed features of both demyelination and axonal degeneration (Nelis et al. Neurology 59(12): 1865-1872, 2002; Ammar et al. Neuromuscular Disorders 13: 720-728, 2003).

Hereditary sensory neuropathy type I (HSNI) is an autosomal dominant ulcero-mutilating disorder of the PNS characterised by progressive sensory loss. The HSNI locus maps to chromosome 9q22.1-22.3 and is caused by mutations in the gene coding for serine palmitoyltransferase long-chain base subunit 1 (*SPTLC1*). We reported a novel missense mutation in exon 13 of the *SPTLC1* gene in twin sisters with a severe phenotype (Verhoeven et al. Neurology 62:6: 1001-1002, 2004).

Identification of novel loci

We reported a Spanish family with autosomal dominant CMT2 not linked to the known loci. We performed a genome-wide scan and obtained conclusive linkage with a chromosome 12q13.13 marker. Fine mapping localized this novel CMT2 locus to a 13.2Mb interval at 12q12-q13.3. In addition we excluded the candidate genes advillin (*AVIL*), centaurin gamma 1 (*CENTG1*), RAB5B member RAS oncogene family gene (*RAB5B*) and the desert hedgehog homolog (Drosophila) gene (*DHH*). Since the CMT2 neuropathy in this family represents a novel genetic entity, we designated it CMT2G (Nelis et al. Journal of Medical Genetics 41(3): 193-197, 2004).

Dominant intermediate CMT (DI-CMT) is a genetic and phenotypic variant of classical CMT, characterized by intermediate NCVs and histological evidence of both axonal and demyelinating features. We performed a genome scan in two unrelated intermediate CMT families and found linkage to a novel locus on chromosome 1p34-p35 (DI-CMTC). The functional and positional candidate genes syndecan 3 (*SDC3*) and lysosomal-associated multispanning membrane protein 5 (*LAPTM5*) were excluded for pathogenic mutations. This is the third locus reported for DI-CMT (Jordanova et al. American Journal of Human Genetics 73: 1423-1430, 2003).

Identification of novel disease associated genes

RAB7

Ulcero-mutilating neuropathies are characterised by prominent sensory loss, often complicated by severe infections and amputations of toes and fingers. So far, two loci and one gene have been reported for autosomal dominant ulcero-mutilating neuropathies. HSN I maps to 9q22.1-q22.3 and is caused by mutations in the *SPTLC1* gene. CMT2B is a hereditary motor and sensory neuropathy assigned to 3q13-q22. We found two missense mutations in the small GTPase late endosomal protein gene RAB7, associated with the CMT2B phenotype in distinct families. RAB7 belongs to the Rab family of Ras-related GTPases. These Rab proteins are essential for the regulation of intracellular membrane trafficking. RAB7 is involved in transport between late-endosomes and lysosomes. Recent studies demonstrated that the RAB7 protein is involved in the targeting of glycosphingolipids. The major lipid component of the myelinated nerve is sphingomyelin. Interestingly, the *SPTLC1* gene, mutated in ulcero-mutilating HSN I patients, is involved in the biosynthesis of sphingolipids. However, it is currently unclear how dysfunction of RAB7 causes the sensory and motor neuropathy in CMT2B patients (Verhoeven et al. American Journal of Human Genetics 72: 722-727, 2003).

KIAA1985

We contributed to the identification of the gene for a childhood-onset demyelinating form of CMT associated with an early-onset scoliosis and a distinct Schwann cell pathology. This type, CMT4C, is inherited as an autosomal recessive trait and mapped to chromosome 5q23-q33. By homozygosity mapping we refined the CMT4C locus to a critical region of 1.7Mb and subsequently identified mutations in an uncharacterized transcript, *KIAA1985*, in 12 CMT4C families. We observed distinct protein truncating and missense mutations targeting amino acids conserved

through evolution. In all families, we identified mutations either in the homozygous or compound heterozygous state. The CMT4C gene is strongly expressed in neural tissues, including peripheral nerve tissue. The translated protein defines a new protein family of unknown function with putative orthologues in vertebrates. Comparative sequence alignments indicated that members of this protein family contain multiple SH3 and TPR domains that are likely involved in the formation of protein complexes (Senderek et al. American Journal of Human Genetics 73: 1106-1119, 2003).

ARHGEF10

As a result of a clinical and electrophysiological examination of a patient for vascular problems of the leg, a phenotype of slowed motor and sensory NCVs was discovered. Reduced NCVs is a hallmark of peripheral neuropathies. Subsequent examination identified slowed NCVs in 12 of 39 relatives, indicating an autosomal dominant inheritance of the phenotype. We performed a genome-wide linkage analysis and obtained conclusive linkage with a particular marker on the small arm of chromosome 8q23. Subsequent analysis restricted the candidate region to 1.5Mb, and within this sequence a total of 5 genes were identified. Genomic sequencing resulted in the identification of a heterozygous mutation in the *ARHGEF10* gene that completely co-segregated in the family. *ARHGEF10* encodes a guanine-nucleotide exchange factor for the Rho family of GTPase proteins. Expression analysis of the mouse orthologue *Gef10* was performed in the developing mouse embryo and showed high *Gef10* expression in the peripheral nervous system. Taken together, our results suggested an important role for *ARHGEF10* in nerve-conduction and also in axon myelination, because thin myelin sheets were also detected in a peripheral nerve biopsy of the proband (Verhoeven et al. American Journal of Human Genetics 73(4): 926-932, 2003).

HSP22 and HSP27

Distal hereditary motor neuropathies (distal HMN) are pure motor disorders of the PNS resulting in severe atrophy and wasting of distal limb muscles. In 1996, we mapped the locus for distal hereditary motor neuropathy type II (distal HMN II) on 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. Subsequently we excluded 12 candidate genes (Irobi et al. 2002). We have now identified a missense mutation (K141N) in the small heat-shock 22-kDa protein 8 (encoded by *HSPB8*; also called *HSP22*). We also found a second mutation involving the same lysine residue (K141E) in two smaller families. Interestingly, we found 5 missense mutations in another small heat shock protein encoded by *HSPB1* - also called *HSP27* - to be associated with distal HMN. Furthermore, one of the *HSP27* mutations (S135F) was found in a CMT family of which the locus (CMT2F) maps to 7q11-q21. This is an intriguing finding since in this type of CMT the sensory nerves are also involved. Small heat shock proteins (sHSP) or stress proteins are molecular chaperones induced in response to a wide variety of physiological and environmental factors, and members of this family share a conserved α-crystallin domain. Most of the mutations in HSP22 and HSP27 target amino acids that are critical for the structural and functional integrity of the small HSP αA-crystallin. Our functional studies confirmed that HSP22 and HSP27 are interacting partners and

that this interaction is increased between mutant proteins. Mutant *HSP22* or *HSP27* showed a decreased viability of neuronal cells, and expression of mutant *HSP22* in cultured cells promoted the formation of intracellular aggregates. HSP27 was shown to be involved in the organisation of the neurofilament network, which is important for the maintenance of the axonal cytoskeleton and transport, and mutant HSP27 affects the neurofilament assembly. This indicates that mutations in HSP22 and HSP27 lead to dysfunction of the axon cytoskeleton and axonal transport, relevant for the pathological mechanism in distal HMN. However, it is currently not known how these molecular chaperones act on other proteins involved in the motor neuron function (Evgrafov et al. Nature Genetics 36(6): 602-606, 2004; Irobi et al. Nature Genetics 36(6): 597-601, 2004).

BSCL2

We contributed to the identification of the gene for Silver syndrome. This neuropathy is a rare form of hereditary spastic paraparesis mapped to 11q12-q14 (SPG17) in which spasticity of the legs is accompanied by amyotrophy of the hands and occasionally also the lower limbs. Silver syndrome and most forms of distal HMN are autosomal dominantly inherited with incomplete penetrance and a broad variability in clinical expression. Linkage to SPG17 was confirmed in additional families with a phenotype characteristic of distal HMN or Silver syndrome. After refining the critical region to 1Mb, missense mutations were detected in the gene encoding seipin (*BSCL2*). Null mutations in BSCL2 were previously shown to be associated with autosomal recessive Berardinelli-Seip congenital lipodystrophy. Little is known on the normal function of seipin, but it is an integral membrane protein of the endoplasmic reticulum. Mutant seipin resulted in aggregate formation thereby leading to neurodegeneration in distal HMN or Silver syndrome (Windpassinger et al. Nature Genetics 36(3): 271-276, 2004). We reported the clinical features of two families with heterozygous BSCL2 mutations. Interestingly, both families show a clinical phenotype different from classical Silver syndrome, and in some patients the phenotype is also different from distal HMN V. Patients in the first family had marked spasticity in the lower limbs and very striking distal amyotrophy that always started in the legs. Patients in the second family had distal amyotrophy sometimes starting and predominating in the legs, but no pyramidal tract signs. These observations broaden the clinical phenotype of disorders associated with BSCL2 mutations, having consequences for molecular genetic testing (Irobi J et al. Nature Genetics 36(6): 597-601, 2004).

SETX

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. Brain 125: 1320-1325, 2002). To identify the molecular

basis of ALS4, we contributed to the study of 19 genes within the ALS4 interval and detected missense mutations (T3I, L389S, and R2136H) in the senataxin gene (*SETX*). Although its function remains unknown, SETX contains a DNA/RNA helicase domain with strong homology to human RENT1 and IGHMBP2, two genes encoding proteins known to have roles in RNA processing. These observations of ALS4 suggest that mutations in SETX may cause neuronal degeneration through dysfunction of the helicase activity or other steps in RNA processing (Chen et al. American Journal of Human Genetics 74(6): 1128-1135, 2004).

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3. Idiopathic 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 aetiology 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.

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.

Mutation analysis of known genes and genotype-phenotype correlations

We previously 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. These findings demonstrated for the first time that *de novo* mutations can cause epilepsy and also that different mutations in the same gene (*SCN1A*) can cause different epilepsy phenotypes (SMEI versus GEFS+). To further investigate the contribution of *SCN1A* mutations to the aetiology of SMEI, we examined nine additional SMEI patients. We observed eight coding and one non-coding mutation. In contrast to our previous study, most mutations are missense mutations clustering in the S4-S6 region of SCN1A. These findings confirm that de novo mutations in *SCN1A* are a major cause of isolated SMEI (Claes et al. Human Mutation 21: 615-621, 2003). They also demonstrate that the nature and the localization of the mutations correlate with the phenotype.

Further we analysed the genes *SCN1B, SCN2A, SCN2B,* and *GABRG2* 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 61(6): 854-856, 2003,). 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*.

Benign familial neonatal convulsions (BFNC) is a rare epileptic syndrome characterized by unprovoked seizures during the first weeks of life with spontaneous remission after a few months. We performed a mutation analysis of KCNQ2 and KCNQ3 in 6 patients of whom 4 had no family history of neonatal seizures. We identified 3 KCNQ2 mutations in 4 patients which all arose de novo. This observation demonstrated that the analysis of KCNQ2 could be useful in selected patients with benign neonatal seizures (Claes et al. Neurology 63(11): 2155-2158, 2004).

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.

Familial temporal lobe epilepsy and febrile seizures belong to the group of idiopathic epilepsies. They sometimes co-occur in families as the variable intrafamilial expression of a presumably monogenic defect. Molecular genetic studies have so far only provided evidence for a digenic inheritance implicating chromosomes 18qter and 1q25-q31 in a single pedigree. In an extended pedigree that was previously excluded for linkage to these loci we performed a 10 cM density genome-wide scan and obtained conclusive linkage (Z_{max} = 6.94 at θ = 0.00) with markers on chromosome 12q23. Fine mapping and haplotype segregation analysis narrowed down the candidate region to a 10.35 cM (8.7 Mb) interval between D12S101 and D12S360. The chromosome 12q22-q23.3 locus represents a novel locus for autosomal dominant familial temporal lobe epilepsy with febrile seizures (Claes et al. Journal of Medical Genetics 41(9): 710-714, 2004).

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Final Report of the Research Group of

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Functional analysis of a gene for progressive hearing impairment

Introduction

The inner ear can be subdivided into two structural and functional components: the vestibular apparatus, which detects accelerations, and the cochlea, which is sensitive to sound. The sensory epithelium of the auditory system is the organ of Corti, a highly specialized and extremely sensitive apparatus. The organ of Corti transduces the mechanical energy resulting from the transmission of a sound wave into an electrical stimulus that is transmitted to the nervous system. After a decade of intense labour and interaction between molecular geneticists and inner ear neuroscientists, some light is being shed on the molecular mechanisms responsible for the complex process of hearing and the pathology of hearing loss.

Our research team contributed to this amazing progress by, among other things, the identification of *DFNA5*, a gene for a non-syndromic, autosomal dominant, progressive, sensorineural (i.e. the defect is located in the cochlea or in the auditory nerve) hearing impairment. The hearing loss starts at the high frequencies at an age between 5 and 15 and progressively affects all frequencies. 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). Expression of the DFNA5 gene could be demonstrated in all tissues investigated, including in the cochlea. Despite extensive computational analyses no putative physiological function, or subcellular localization for DFNA5 could be deduced.

This project concerns the functional characterization of *DFNA5*. More generally, we wish to acquire insight into the molecular mechanisms leading to hearing impairment.

A yeast model for the study of human DFNA5

Mcm10 is required for the efficient initiation of DNA replication and might also be required for the elongation step of DNA replication. All known members of the Mcm10 family share a conserved zinc-finger-like motif, referred to as the CCCH domain, which is essential for the Mcm10 function. We used PROSITE to search SWISS-PROT, TrEMBL and PDB databases using a consensus sequence of the CCCH domains of Mcm10 proteins. Apart from known Mcm10 homologues, we identified the human DFNA5 protein and its mouse homologue Dfna5h. Interestingly, the mutation associated with non-syndromic hearing loss results in a truncated protein that is lacking the region containing the CCCH domain. To further characterize the DFNA5 proteins, we identified two additional homologues: rat and horse Dfna5. Both these DFNA5 homologues share the domain of homology with the Mcm10 family (Figure 1) (Gregan et al., 2003).

Subsequently, we cloned wildtype alleles of mouse and human *DFNA5* as well as the mutant *DFNA5* allele from DFNA5 patients and expressed them in the fission yeast Schizosaccharomyces pombe under control of the regulatable *nmt1* promoter. The *nmt1* promoter can be repressed in the presence of thiamine; it can be induced by removing thiamine. While heterologous

expression of human and mouse DFNA5 proteins did not result in any apparent phenotype, expression of the mutant DFNA5 allele caused loss of viability of the yeast cells as assessed from growth on plates lacking thiamine (Figure 2) (Gregan et al., 2003).

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                                 :  .  :   * *    :     *                .              *
mcm10_human              VLIMGEALDLGTCK-AKKNGEPCTQTVNLRD-CEYCQYHVQAQYKKLSA-KRADLQ(377-430)
mcm10_Xenopus            VLLMGDAVDLGTCK-ARKNGDPCTQMVNLND-CEYCQYHVQAQYKKVSS-KRADLQ(369-422)
mcm10_Drosophila         VMILGQSKDLGTCR-ATKKNGDKCTSVVNLTD-CDYCIFHVKQEYGKMS--RRSELQ(124-176)
mcm10_Anopheles          VMVLGQSRDLGTCR-SRKKNGDRCTSIVNLGK-CEYCVYHIKQEYNKAS--NRGGLL(196-249)
mcm10_S.cerevisiae       ILEIGSSRDLGWCPIVNKKTHKKCGSPINISL-HKCCDYHREVQFRGTSA-KRIELN(297-351)
mcm10_C.elegans          IVEIGQSAHFGTCKGIRQQDGQRCSNFVNSSL-SEFCVFHVMSAARKLSA-KRGVFN(254-308)
Dfna5_human              EMPDSAAALLGTCC-KLQIIPTLCHLLRALSD-DGVSDLEDPTLTPLKDT-ERFGIV(395-448)
Dfna5_horse              EMPDNAAALLGTCC-KLQIIPALCHLLHAMSH-DGVCDLEDPALAPLKDT-ERFGVA(396-449)
Dfna5h_mouse             EMPDNATVFLGTCC-KLHVISSLCCLLHALSD-DSVCDFHNPTLAPLRDT-ERFGIV(398-451)
Dfna5_rat                EMPDNATVFLGTCC-KLHVIPSLCHLLHALSD-DSVCDFQDPTLAPLRDT-ERFGIV
mcm10_S.pombe            LLEIGRSKHLGYCS-SRRKSGELCKHWLDKRA-GDVCEYHVDLAVQRSMS-TRTEFA(287-340)
mcm10_Arabidopsis        MVKLGVSADYGVCT-AKRKDGTTCTSVVNKNLRTAFRDLKSQGIYTVEPPADRSGNK(198-253)

consensus/90%            .h..stsh.hGhCp..hph.sp.Cp.hhshp..sths.hc....h.h.s..pR.th.
```

Figure 1. DFNA5 and Mcm10 proteins share a domain of homology. Multiple alignment of the Mcm10-CCCH domains of human, Xenopus laevis, Drosophila melanogaster, Caenorhabditis elegans, Anopheles gambiae, Arabidopsis thaliana, Schizosaccharomyces pombe and Saccharomyces cerevisiae and of DFNA5 proteins from human, mouse, horse and rat aligned using CLUSTALW. Identical residues are indicated by an asterisk, strong and weak conservations based on CLUSTAL Ware indicated by a double dot and single dot respectively. The first and the last aligned residues in each of the sequences are designated on the right. The 90% consensus shown below the alignment was calculated using Consensus software (http://www.bork.embl-heidelberg.de:8080/Alignment/consensus.html) and the following amino acid groupings were used: hydrophobic (h; A,C,F,G,H,I,K,L,M,R,T,V,W,Y), small (s; A,C,D,G,N,P,S,T,V), turnlike (t; A,C,D,E,G,H,K,N,Q,R,S,T), polar (p; C,D,E,H,K,N,Q,R,S,T), charged (c; D,E,H,K,R).

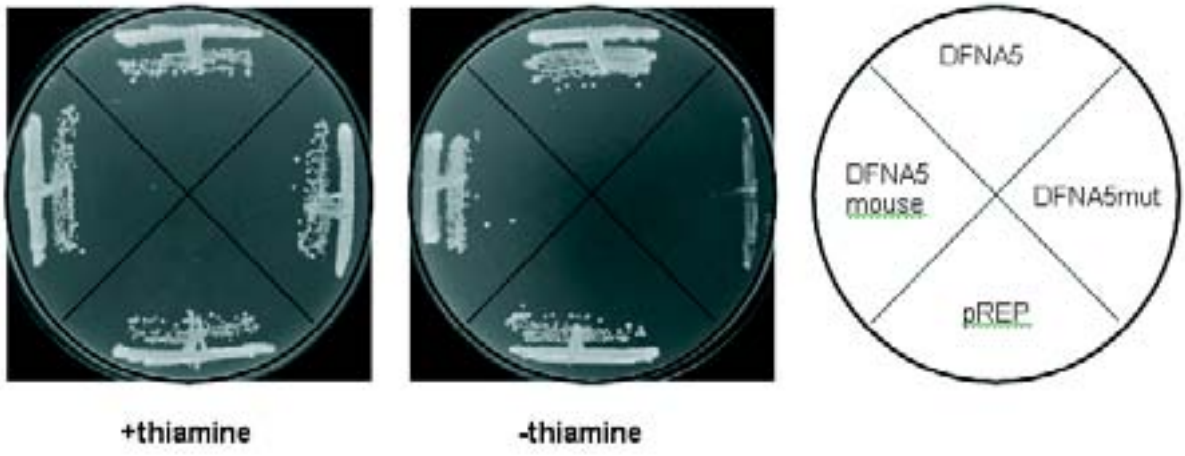

Figure 2. Heterologous expression of DFNA5 proteins in yeast. Wild-type strain (P138) transformed with either empty vector pREP3x (pREP), or a construct containing mouse Dfna5h (DFNA5mouse), human wild-type DFNA5 (DFNA5) or the human mutant allele (DFNA5mut) were grown on EMM plates with or without added thiamine.

Additional families with DFNA5 mutations

The extended Dutch family, in which DFNA5 first was identified, long remained the only DFNA5 family. This has changed recently, with the description of a Chinese family harbouring a 3-nucleotide deletion in the polypyrimidine tract of intron 7 (Yu et al., 2003) and a second Dutch family (Bischoff et al., 2004) with a nucleotide substitution in the splice-acceptor site of intron 7. In general, the hearing loss in the newly described families is very similar to that found in the original Dutch family.

Formulation of a gain-of-function hypothesis

At the genomic DNA level, the mutations in DFNA5 leading to hearing loss are diverse (Van Laer et al., 1998; Yu et al., 2003; Bischoff et al., 2004). Most interestingly, however, at the mRNA level, all of these mutations lead to skipping of exon 8, which indicates that only this particular event might cause hearing impairment. It cannot be a coincidence that, despite being different at the level of DNA, all three currently known mutations of DFNA5 lead to skipping of exon 8. Mutations in other parts of DFNA5 might have no clinical effect at all or might result in a completely different phenotype. The fact that skipping of one specific exon in a gene leads to a particular monogenic phenotype that is not caused by other mutations in this gene is a highly unusual finding and, to the best of our knowledge, unprecedented. This fact, in combination with the fact that no other mutations in other parts of the gene have been described, led to the formulation of the hypothesis that DFNA5 associated hearing loss is caused by a gain-of-function mutation. A first line of evidence supporting the hypothesis of a deleterious new function for mutant DFNA5 was described above: we demonstrated that the expression of human mutant DFNA5 in yeast cells caused loss of viability of the yeast cells, while expression of human wildtype DFNA5 did not harm the yeast cells (Figure 2).

Further evidence for our hypothesis that human mutant DFNA5 might exert a deleterious new function was gathered during experiments originally initiated to elucidate the subcellular localisation of wildtype and mutant DFNA5. PSORTII predicted the most probable subcellular localisation for DFNA5 to be cytoplasmic, while prediction of transmembrane regions with PSORTII and Tmpred (http:// www.ch.embnet.org/software/TMPRED) resulted in a varying number of transmembrane regions with relatively low scores. To test these predictions experimentally, we tagged wildtype and mutant DFNA5 with GFP, transfected the constructs into COS-1 and HEK293T cell lines, and analysed cell death with flow cytometry and fluorescence microscopy. Wildtype DFNA5-GFP was distributed smoothly in the cytoplasm, while mutant DFNA5-GFP was localised in the cytoplasm in a granular manner. In addition to its cytoplasmic localisation, mutant DFNA5-GFP seemed to be associated with the plasma membrane in HEK293T cells, especially in cells with an unhealthy appearance (a more circular shape rather than a stretched shape and with cell blebbing). Cell death of mutant DFNA5-GFP transfected cells was confirmed with flow cytometry (Figure 3). Even when we took into account cell death because of trypsinisation and lipofectamine, post-transfection cell death approximately doubled when cells were transfected with mutant DFNA5-GFP compared with wildtype DFNA5-GFP (Van Laer et al., 2004).

As DFNA5 has been implicated in signalling pathways that lead to apoptosis, we investigated whether the observed cell death was the consequence of apoptotic or necrotic events using a double staining procedure with Hoechst 33342 and ethidium bromide. We could demonstrate that cell death was attributed to necrotic events (Van Laer et al., 2004).

We concluded that the transfection experiments in mammalian cell lines support our hypothesis that the hearing impairment associated with DFNA5 is caused by a "gain of function" mutation and that mutant DFNA5 has a deleterious new function. Further experiments are needed to elucidate the exact pathological mechanisms that lead to cell death in cells transfected with mutant DFNA5. These experiments might shed light on the pathology that leads to hearing loss in families with DFNA5 mutations.

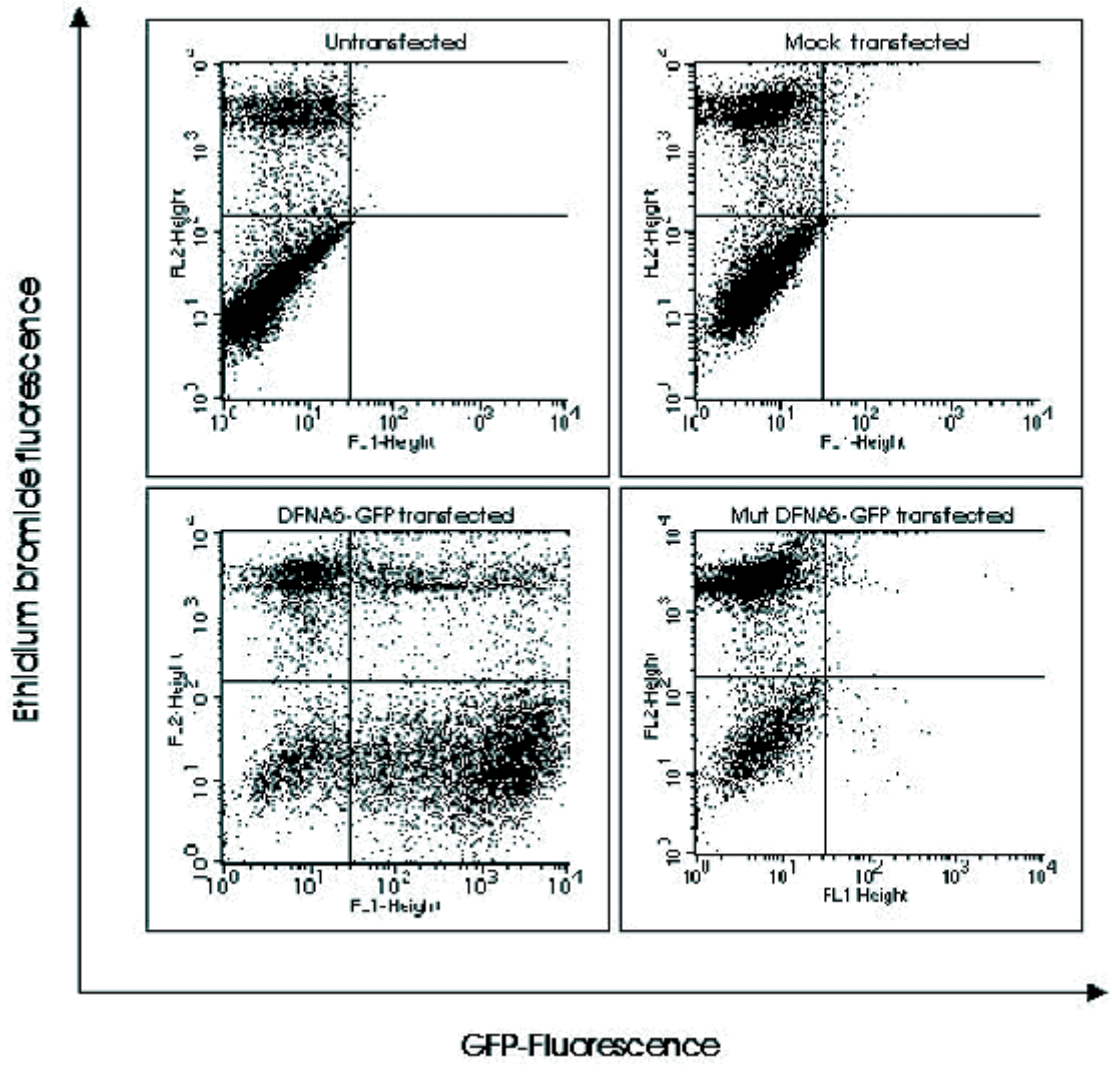

Figure 3. Transfection experiments in HEK293T cells. Twenty-four hours post-transfection, HEK293T cells were trypsinised and studied in the presence of ethidium bromide using FACScan flow cytometry. In the dot plots, the lower left quadrant represents viable GFP- cells, the lower right viable GFP+ cells, the upper left dead GFP- cells, and the upper right dead GFP+ cells. Similar results were obtained using COS-1 cells (not shown).

Investigation of *DFNA5* as putative Age-Related Hearing Impairment (ARHI) gene

As the most frequent type of ARHI is progressive, sensorineural and most pronounced in the high frequencies, genes causing monogenic hearing impairment with phenotypic similarities to ARHI, *DFNA5* for example (although with a much younger age-at-onset), are excellent candidate ARHI susceptibility genes.

We performed linkage analysis to a quantitive measure of high frequency hearing loss with microsatellite markers from the DFNA5 region. No significant linkage could be detected. Subsequently, the DFNA5 coding region was analysed for single nucleotide polymorphisms (SNPs), resulting in the detection of 6 SNPs. Two SNPs leading to amino-acid substitutions (P142H and V207M) were selected for further analysis. A pilot experiment on 116 random Belgian and Dutch subjects was initiated, but no association could be detected. Subsequently, 93 ARHI cases and 83 controls, selected from the Framingham cohort, were genotyped for P142H. Again, no significant differences in genotypes between good hearing and hearing impaired individuals could be detected. In conclusion, this study could not detect a significant association using moderate sample sizes, suggesting the absence of a major effect (Van Laer et al., 2002).

Characterization of a DFNA5 knockout mouse

To study DFNA5 function *in vivo*, Dfna5 knockout mice were generated through the deletion of exon 8 by targeted recombination in 129 ES cells, simultaneously mimicking the human mutation. As the 129 strain exhibits hearing loss itself, the genetic background was changed by crossing for 10 generations with C57Bl/6J, a strain with late-onset hearing loss, and with CBA/Ca, a good-hearing reference strain. After 10 generations of breeding, 99.9 % of the genome was derived from either C57Bl/6J or CBA/Ca.

Using RT-PCR on brain mRNA we could demonstrate the presence of mutant *Dfna5* transcript in heterozygous and homozygous Dfna5 mice. However, using Western blotting we could not detect Dfna5 protein in brain tissue derived from homozygous animals, indicating that the *Dfna5* gene was effectively silenced.

To test the hearing impairment, frequency-specific ABR (Auditory-evoked Brainstem Response) measurements were performed at different ages in both genetic backgrounds, but no differences between Dfna5-/- mice and their wildtype littermates could be demonstrated for any age or either genetic background (Figure 4). In addition, we could not detect significant differences in vestibular function between Dfna5-/- and Dfna5+/+ mice using a series of simple tests (swimming test, elevated platform test, reaching response, negative geotaxis, circling behaviour, air righting reflex and contact righting reflex). In view of the results obtained previously with the ABR analysis, it was decided to discontinue vestibular testing and not to proceed with older ages, a second genetic background (CBA/Ca) or vestibulo-ocular reflex (VOR) and optokinetical reflex (OKR) tests.

Scanning electron microscopical examinations revealed significant differences between Dfna5-/- and Dfna5+/+ mice in the number of fourth row outer hair cells. Outer hair cells normally occur in 3 carefully arranged rows. Some mice however, show stretches of 4th row outer hair cells and a disturbance of the pattern. Significant results were obtained in both genetic backgrounds, albeit with opposite effects. In the C57Bl/6J background, Dfna5-/- mice showed an increased number of fourth row outer hair cells when compared to Dfna5+/+ mice, while in the CBA/Ca background the number of fourth row outer hair cells was reduced (Figure 5). The scanning electron microscopical results were obtained with adult mice. Another technology, namely the preparation of explants of the organ of Corti, was used to investigate whether the differences in number of outer hair cells were already present in newborn mice, which would indicate that these cells develop together with the other inner and outer hair cells. At least in the C57Bl/6J genetic background, the electron microscopical results could be confirmed in the middle portion of the cochlea.

As *Dfna5* expression could be demonstrated in every tissue investigated so far, a general histopathological examination was performed at macroscopical and microscopical level of 46 organs, including the ear. In addition, an external examination and an X-ray analysis were performed and a chemogram, hemogram, and urine profile were generated. This extensive examination confirmed that the middle and inner ear of the Dfna5-/- mouse have a normal structure, and that at the light microscopical level no differences can be observed between Dfna5-/- and Dfna5+/+ mice in the organ of Corti and the cochlear ganglion cells. No additional abnormalities were seen in any of the other 44 organs examined in the Dfna5-/- mouse. In addition, X-rays were considered normal, and blood and urine values were within normal range.

In Dfna5 knockout zebrafish, a complete absence of UDP-glucose dehydrogenase (Ugdh) and a strong reduction in hyaluronic acid (HA) levels were demonstrated in the developing ear and pharyngeal arches (Busch-Nentwich et al., 2004). Therefore, we investigated the presence of *Ugdh* mRNA and HA in Dfna5 knockout mice with quantitative real-time PCR and hyaluronic acid histochemistry, respectively. Relative *Ugdh* levels were measured in inner ear derived from newborn Dfna5-/- and Dfna5+/+ mice, but no significant differences were obtained. An identical HA expression pattern was observed in Dfna5+/+ and Dfna5-/- mice, with no difference in staining intensity (Figure 6).

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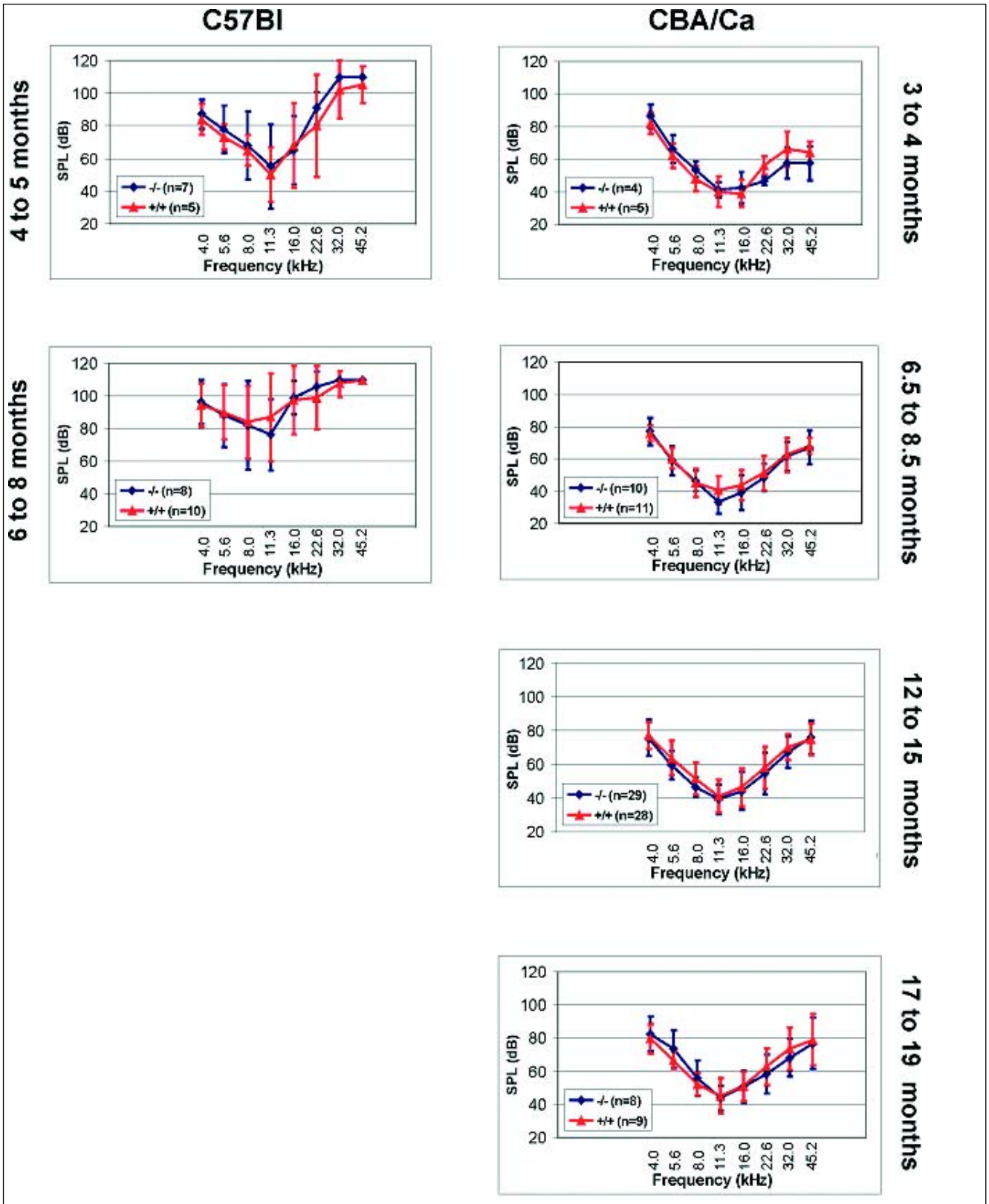

Figure 4. Frequency-specific auditory-evoked brainstem response (ABR) of Dfna5 knockout (-/-) mice and their wild-type littermates (+/+) in two genetic backgrounds. Mice of the C57Bl/6J genetic background were tested at an age of 4 to 5 months (143 ± 16 days) and 6 to 8 months (210 ± 22 days). Mice of the CBA/Ca genetic background were tested at an age of 3 to 4 months (101 ± 12 days), 6.5 to 8.5 months (221 ± 26 days), 12 to 15 months (399 ± 31 days) and 17 to 19 months (541 ± 23 days). The X-axis indicates the different test frequencies, while the Y-axis indicates the sound pressure level (SPL). The results from 2 and 2.8 kHz were omitted from the figure.

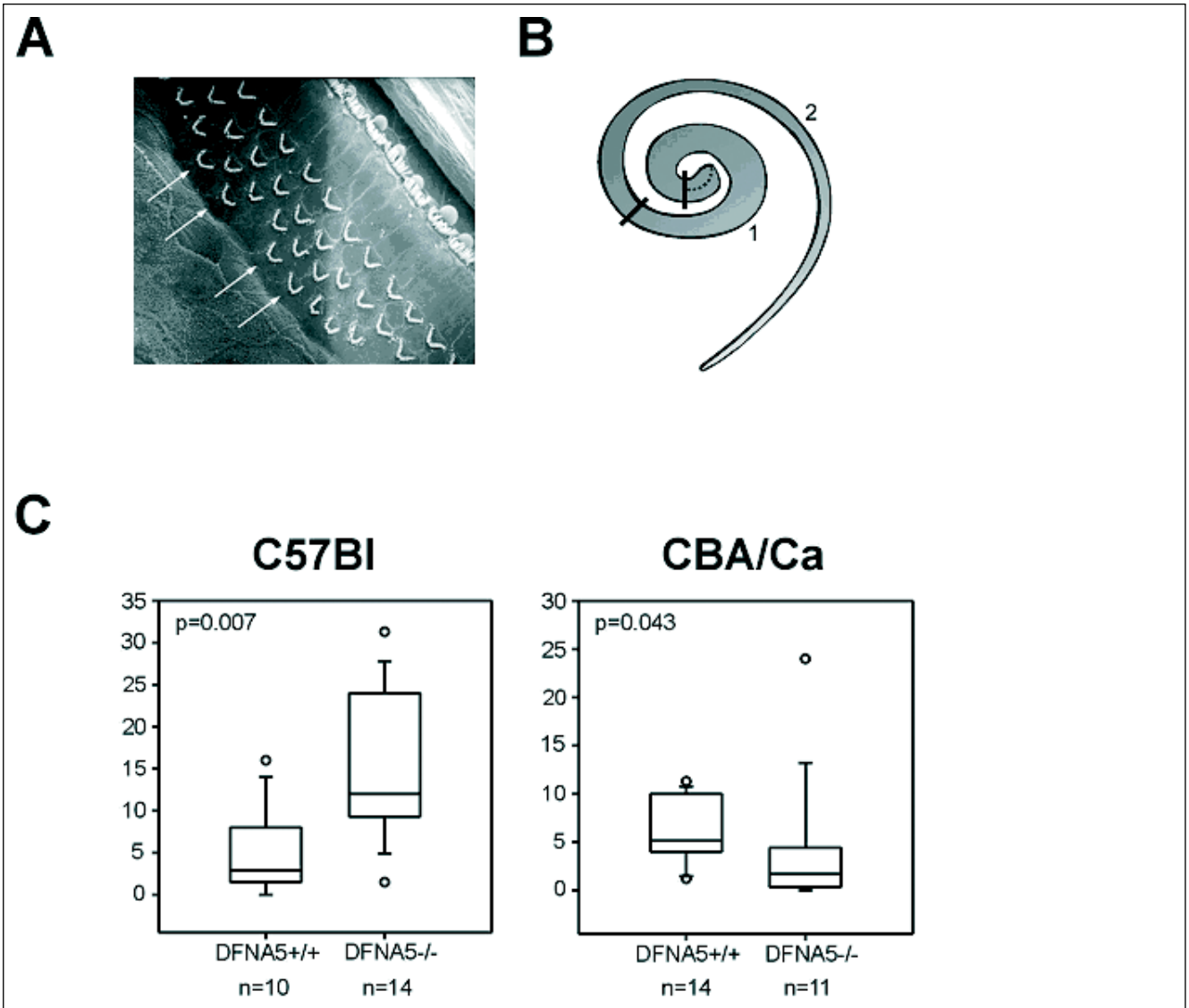

Figure 5. Scanning electron microscopy of cochlear hair cells in the organ of Corti. A typical result is presented in panel (A). The arrows indicate supernumerary (fourth row) outer hair cells, which were counted along the cochlear turns. (B) A diagram representing the division of the cochlea, for counting purposes, in an apical turn (turn number 1) and a basal turn (turn number 2). The portion near the helicotrema is indicated with a dotted line and was omitted from counting, as the hair cell pattern is very irregular in this cochlear part. (C) Counts of fourth row outer hair cells in the apical turn of the cochlea in DFNA5-/- mice and their wild-type littermates. The rectangles of the box plots represent the 25% to 75% percentiles; the median is indicated as well. The 10% to 90% percentiles are given by the thin lines. All values outside the 10% to 90% percentiles are represented by small circles.

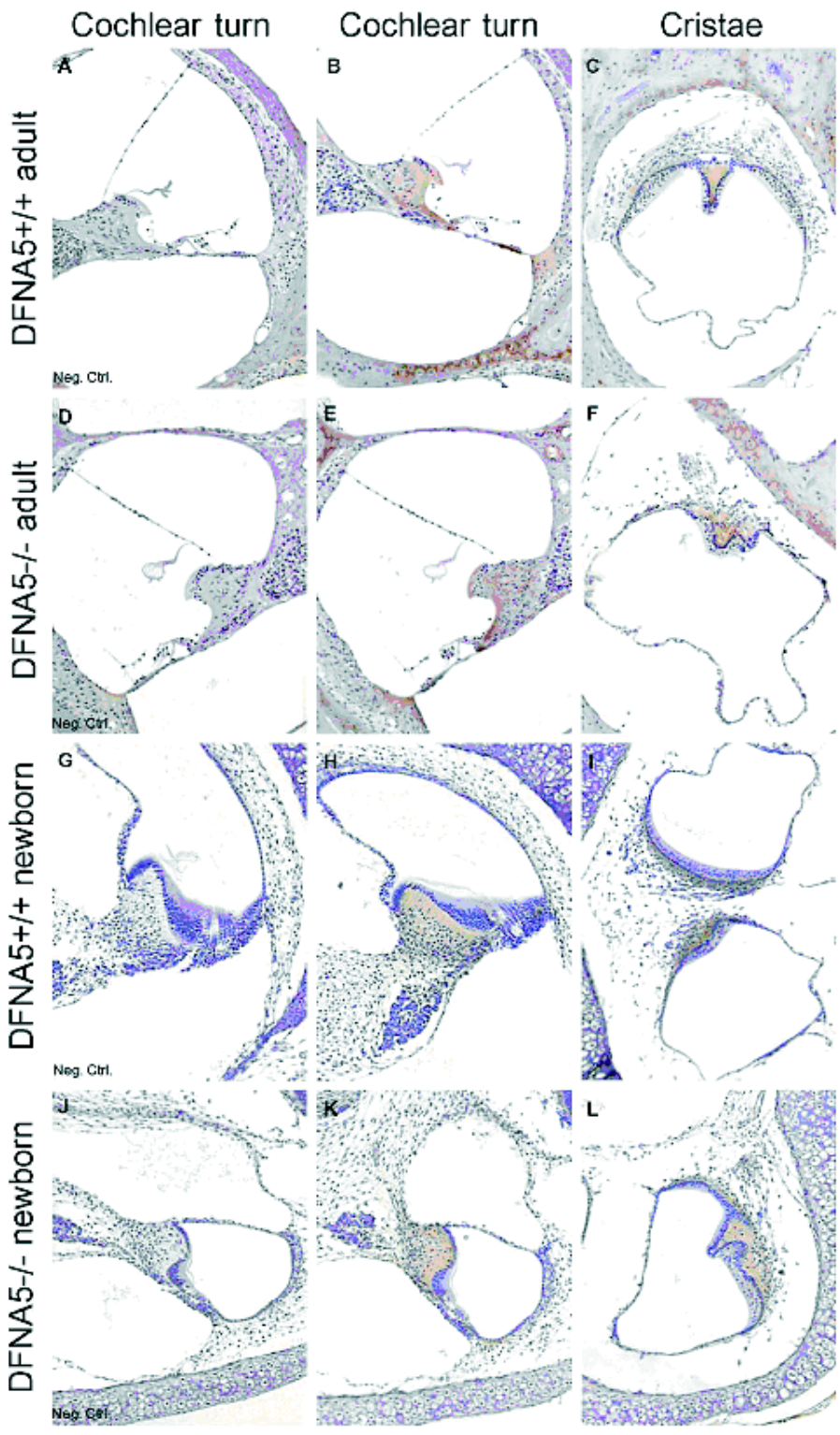

Figure 6. Hyaluronic acid staining of Dfna5 knockout (-/-) and wild-type (+/+) inner ears at two different ages (adult and newborn). For each stage a cochlear turn (B and E for the adult stage, H and K for the newborn stage) and the cristae (C and F for the adult stage, I and L for the newborn stage) are shown for Dfna5+/+ (B, C, H, and I) and Dfna5-/- (E, F, K and L) mice. In addition, for each cochlear turn a corresponding negative staining without B-HABP is given (A, D, G and J). The negative control for the cristae staining is not shown.

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Publications relevant to the programme (*)

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

A set of left-hemispheric regions has been consistently activated in a wide variety of different language tasks compared to non-language control conditions (for review see [13] [19] [20]). In this project we have dissected the differential contributions of these regions to three closely related cognitive functions: Naming, reading or knowing. Our convergent approach consisted of a combination of psychophysical and functional anatomical studies in normal ageing, incipient Alzheimer's disease and primary progressive aphasia.

As outlined in the original proposal, naming and reading share many component processes: Retrieval of the internal representation of the word (lexical-retrieval), retrieval of its meaning (conceptual or semantic retrieval) and of what the word sounds like (phonological retrieval) [12] [23]. Naming and reading however differ by the gate through which the language system is entered and by the pathway followed through this system [16]. In the original proposal we put forward an a priori neuroanatomical model. In this hypothetical model some areas established a link between different sorts of internal representations:

1. The ventral occipitotemporal junction and the posterior inferior temporal gyrus (Brodmann area (BA) 37) were thought to be involved in linking the structural representation of a visual object to the internal representation of its name (from object to name and from name to object).
2. The posterior end of the inferior temporal sulcus was a gate of entry from the structural representation of a visual object into the conceptual system [26].
3. The middle third of the superior temporal sulcus, the left inferior frontal sulcus and BA 47 were gates of entry from the internal representation of a word into the conceptual system [26].

According to the original model, the anterior and the medial temporal cortex functioned as associative structures encoding and retrieving relationships between conceptual nodes that have a distributed cortical representation [25] [26]. The dorsolateral prefrontal cortex was involved in searching the semantic web. Two classical language areas, the frontal operculum and the posterior middle temporal gyrus, were proposed to be involved in retrieving and encoding phonological representations [20] [13], processes that lay outside the focus of this project. Each part of our a priori model has been tested and our integrative approach has provided reasons for major modications of the model as it was outlined in the original 2001 proposal.

1 Input-modality specific gates of entry into the semantic system

1.1 Pictures

In a group of 30 cognitively intact elderly individuals, aged between 50 and 80 years of age, we conducted a factorial fMRI experiment (Fig.1) [26] [32]. The first factor, task, had two levels: Associative-semantic (Fig.1A panel A, C) versus visuoperceptual judgment (Fig.1A panel B,D). The second factor, input modality, also had two levels: Pictures (Fig.1A panel A,B) or printed words (Fig.1A panel C,D). The associative semantic condition (Fig.1A panel A,C) was derived from the Pyramids and Palm Trees test [8] [7] [6].

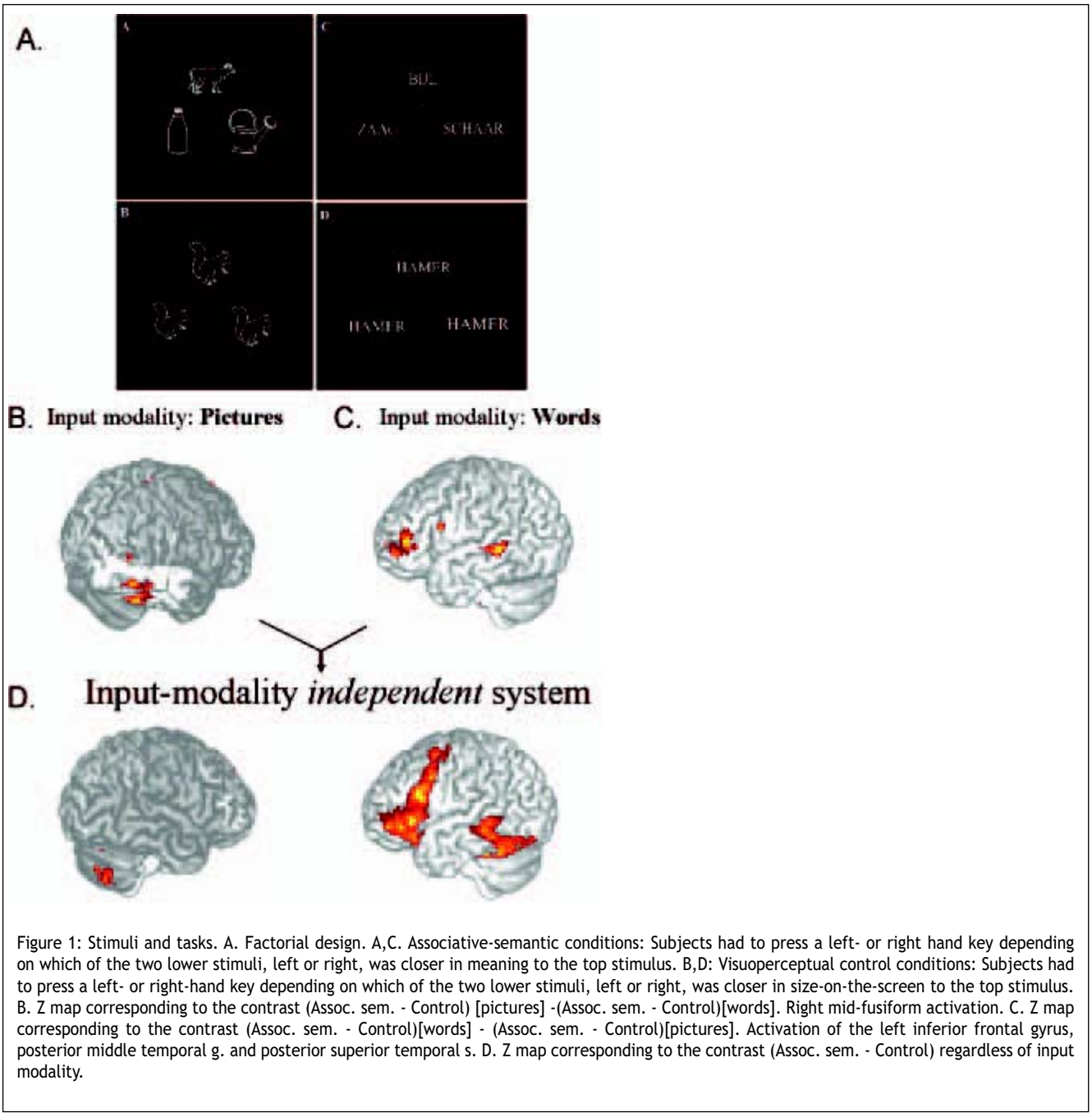

Figure 1: Stimuli and tasks. A. Factorial design. A,C. Associative-semantic conditions: Subjects had to press a left- or right hand key depending on which of the two lower stimuli, left or right, was closer in meaning to the top stimulus. B,D: Visuoperceptual control conditions: Subjects had to press a left- or right-hand key depending on which of the two lower stimuli, left or right, was closer in size-on-the-screen to the top stimulus. B. Z map corresponding to the contrast (Assoc. sem. - Control) [pictures] -(Assoc. sem. - Control)[words]. Right mid-fusiform activation. C. Z map corresponding to the contrast (Assoc. sem. - Control)[words] - (Assoc. sem. - Control)[pictures]. Activation of the left inferior frontal gyrus, posterior middle temporal g. and posterior superior temporal s. D. Z map corresponding to the contrast (Assoc. sem. - Control) regardless of input modality.

During a trial, a triplet of stimuli was presented for 5350 ms, one stimulus on top (the sample stimulus) and one in each lower quadrant (the test stimuli) (Fig.1A panel A, C), followed by a 1500 ms interval. Subjects had to press a left- or right-hand key depending on which of the two test stimuli matched the sample stimulus most closely as for its meaning. A given triplet was presented in either the picture or the word format and this was counterbalanced across subjects. In the visuoperceptual control condition (Fig.1A panel B,D), a given stimulus was presented in three different sizes. Subjects had to press a left or right-hand key depending on which of the two test stimuli matched the sample stimulus most closely as for its size on the screen. The difference in size between distractor and target was 9% and the difference in size beteen target and sample stimulus was also 9%. The size of the sample stimulus varied across trials.

In addition to the originally described activation pattern [26], the right mid-fusiform g. (36,-51,-21, Z=5.02) was more active when subjects performed an associative-semantic task with pictures than when they performed a visuoperceptual task (Fig.1B,Fig. 2B)). This activation was not present when the input consisted of words (interaction between modality and task) [32].

We carried out a detailed neuropsychological and psychophysical assessment of a patient [32] (Fig. 2). The mid-fusiform area of activation described in the previous paragraph (Fig.1B) coincided with the anterior portion of her lesion (Fig. 2A). The patient was impaired on the following tasks: Pseudo-object decision (BORB) [22], identification of objects from silhouettes (VOSP) [37], the Rey Visual Design Learning test [21] (RVDLT) (episodic memory for abstract shapes) and the Benton Visual Retention test (BVRT) (short-term memory for abstract shapes), as well as discrimination of 3D from texture. When words or pictures were presented foveally and stimulus durations ranged from 30 to 800 ms, the patient showed significant impairment on word reading and picture naming at short stimulus durations. Impairment of all of these tasks could still be attributed to a higher-order visuoperceptual deficit. However, drawing common objects from memory was strongly impaired while she was able to copy figures in great detail (Fig. 2D). She was also impaired on verbal associative semantic tasks under the condition where the concepts and the links were highly imageable [10]. These two deficits cannot be explained by visual impairment and point to impaired retrieval of mnemonic features of objects.

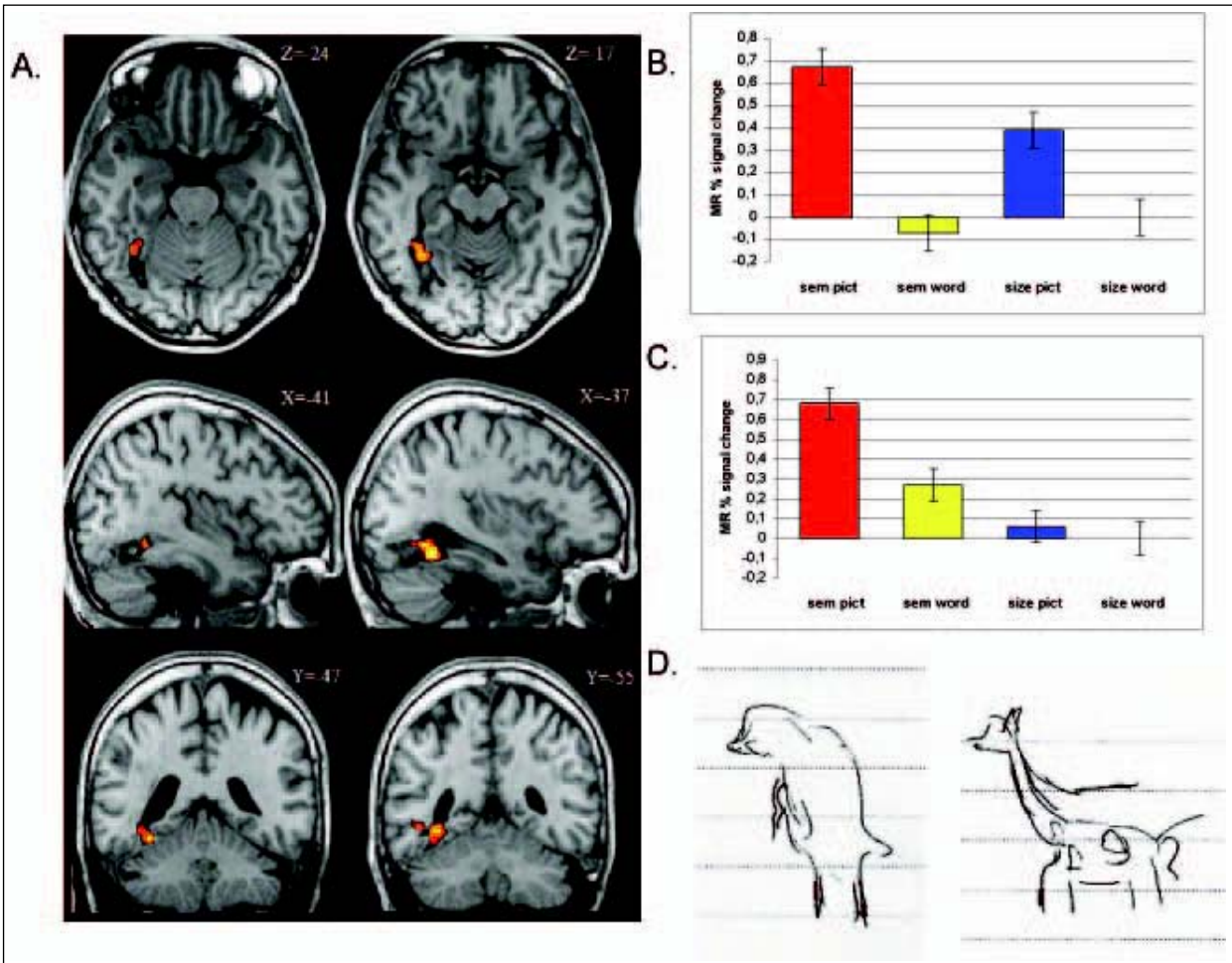

Figure 2: A. Superposition of the activation focus obtained in normal controls when the associative-semantic condition with pictures is compared to the visuoperceptual condition onto the structural MRI of the patient. The lesion of the patient can be seen as a hypointensity in the right fusiform g. B. Activity prole in the normal controls in the right mid-fusiform g. Legend: Red: Associative semantic condition with pictures. Yellow: Associative semantic condition with words. Blue: Visuoperceptual condition with pictures. Baseline: Visuoperceptual condition with words. Activity during the associative-semantic condition with pictures is significantly higher than activity during the visuoperceptual condition with pictures while no increase is seen when the same conditions are compared with words. C. Activity prole in the normal controls in the symmetrical left-sided mid-fusiform g.: Activity is increased during the associative semantic condition with pictures compared to the visuoperceptual condition with pictures but also when the same tasks are compared with words. D. Drawing from memory on verbal command: Left: Kangooroo, Right: Girae. In contrast with spontaneous drawing, the patient was able to copy gures in great detail (not shown).

We conclude that the right mid-fusiform gyrus mediates higher-order visuoperceptual processing and also retrieval of stimulus features from memory. The type of retrieved features cut across the divide between short-term (BVRT), episodic (RVDLT) and semantic memory. They pertain to learned features of abstract shapes as in the RVDLT and the BVRT or to overlearned and shared knowledge of object attributes as required during spontaneous drawing or associative tasks with highly imageable concepts (PALPA).

1.2 Words

When subjects performed an associative-semantic compared to a visuoperceptual task with words, the left inferior frontal gyrus and left posterior middle temporal gyrus and posterior superior temporal sulcus were more active. This activity was not present when the same tasks were compared with pictures as input modality (Fig. 1 C).

1.2.1 Word processing in amnestic mild cognitive impairment (MCI)

We studied a group of 14 patients with amnestic MCI, according to the Petersen et al. criteria [18] [30] [35]. The patients had an isolated episodic memory deficit without impairment of other cognitive domains on clinical or standard neuropsychological examination. We conducted a psychophysical experiment in 10 of the MCI patients and 10 of the controls in whom we determined time-accuracy curves (Fig. 3A,B) [36] as well as voice onset latencies during picture naming and word reading. A trial consisted of a forward mask (200 ms duration), followed by a test stimulus immediately followed by a backward mask (200 ms duration), with an inter-trial interval of 5000 ms. Stimuli were presented with variable stimulus presentation durations: 30, 45, 60, 90, 150, 200, 500 or 800 ms. The test stimulus could be a word or a picture. Subjects were instructed to read the word or name the picture. Responses were recorded and voice key latencies were determined digitally (Adobe Audition 1.5, Adobe Systems Incorporated, CA, USA). For each individual, accuracies were analysed using the following exponential equation [36]:

$$p = c(1 - e^{[(a-t)/b]})(1)$$

In this equation, p corresponds to the percentage of correctly identified items, t to the stimulus duration, a corresponds to the onset of the rising curve, b is a measure of the rate with which performance rises to its maximum level and c is the maximum level of performance (asymptote). Parameters a, b and c were subsequently analyzed with a 2x2 factorial ANOVA with modality as within-subject factor and group as between-subject factors. Planned comparisons of Least Square means were used for posthoc analysis. The slope (psychophysical parameter b) of the time-accuracy function for word reading differed significantly between controls and MCI ($F(1, 18)=5.5, P<0.05$) (Fig. 3A). The slope for the picture naming task did not differ significantly between the two groups (Fig. 3B), although there was a trend to be less steep for MCI than for controls ($F(1, 18)=1.4, P<0.05$) (Fig. 3B).

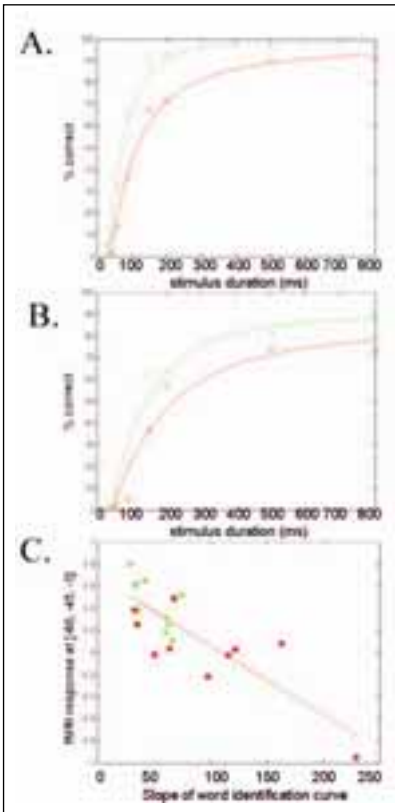

Figure 3: Psychophysical experiment. A. Word reading task. Time-accuracy function. X axis: Stimulus presentation duration. Y axis: % correct responses. Each datapoint corresponds to the average accuracy in the MCI (red) and the control (green) group for a given stimulus duration. B. Picture naming task. Time-accuracy function. Same conventions as in A. C. Correlation between the slope (psychophysical parameter b) of the time-accuracy curve for word reading (A) and the fMRI response in the left posterior middle temporal gyrus (Fig. 7). The larger parameter b is, the less steep the slope.

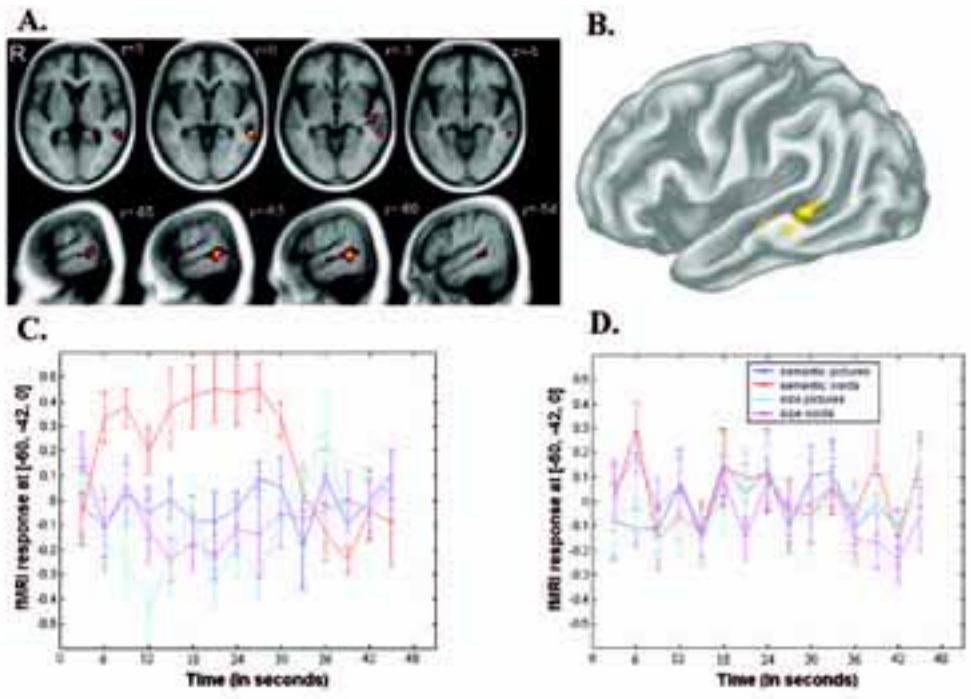

Figure 4: Group results: (Associative-semantic minus visuoperceptual conditions in controls) minus (Associative-semantic minus visuoperceptual conditions in MCI). A. Z map thresholded at P<0.05 corrected and superimposed onto a structural MRI. Upper row: Transverse sections through z levels 3, 0, -3 and -5 mm; Lower row: Sagittal sections through x levels -65, -63, -60 and -54 mm. B. Z map superimposed on a lateral view of a standard brain using CARET software (http://brainmap.wustl.edu). C. Control group. Time course during the four types of epochs (epoch duration=27 s) in the posterior temporal voxel of peak differential activation. Error bars: Standard errors. Red: Associative-semantic judgment with words. Blue: Associative-semantic judgment with pictures. Magenta: Visuoperceptual judgment with words. Cyan: Visuoperceptual judgment with pictures. X axis: Time in s; Y axis: % signal change. D. MCI group. Time course in the same voxel as that plotted in C, averaged over all MCI subjects. Same conventions as in C.

Critically, in one region the BOLD difference between the associative-semantic and the visuoperceptual conditions differed between controls and MCI patients: The posterior third of the left middle temporal gyrus extending into the posterior superior temporal sulcus and anteriorly into the middle third of the superior temporal sulcus (-60,-42,0, ext. 18, Z=4.25, corrected P<0.05) (Fig. 4A,B). In normal controls this region was active during the associative-semantic task with words compared to the visuoperceptual task with words (Z=4.0, corrected P<0.05) (Fig. 4C, red versus magenta) but not when the same tasks were compared with pictures (Z=1.12) (Fig. 4C, blue versus cyan), resulting in an interaction between task and modality (Z=3.29, uncorrected P<0.001). In the MCI group the region was not activated during the associative-semantic task with words compared to the corresponding visuoperceptual task (Fig. 4D, red versus magenta) nor with pictures (Fig. 4D, blue versus cyan), even when we lowered the threshold to P0.05 uncorrected. The difference between the associative semantic and the visuoperceptual condition with words was significantly larger in normals (Fig. 4C, red versus magenta) than in MCI patients (Fig. 4D, red versus magenta) (interaction between group and task -63,-39,0, Z= 4.10, corrected P<0.05) while there was no significant difference between the two groups in this region when pictures were used and the two tasks were compared (interaction between group and task -63,-39,-3, Z=2.0) (Fig. 4C versus D, blue versus cyan). Twelve out of 14 MCI patients had lower activity than their age-matched controls (Fig. 5A). Discriminant analysis revealed good discrimination between groups on the basis of fMRI response in the left superior temporal sulcus (F(1,26)=26.6, P<0.00001): 12 of 14 MCI patients and 11 of 14 normal subjects were correctly classied (sensitivity: 85.7%, specificity: 78.6%). According to a Receiver Operator Characteristic analysis (Fig. 5B), the area under the curve was 0.84. Apart from the posterior temporal cluster, no other brain areas showed significant differential activation between the two groups for any of our a priori contrasts. We did not find any regions where activity was higher in MCI compared to normal controls.

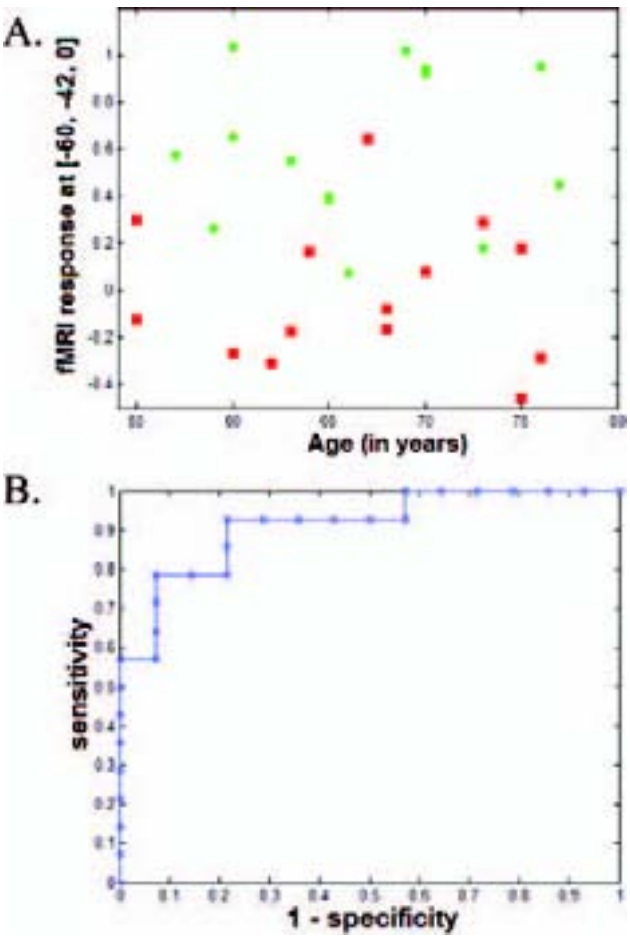

Figure 5: Detailed characterization of the the posterior temporal voxel of peak differential activation between MCI and controls A. Individual BOLD responses. X axis: Age; Y axis: Percentage signal change. Green: Controls. Red: MCI. B. Receiver Operating Characteristic curve: X axis: 1-specificity; Y axis: Sensitivity. The different datapoints correspond to stepwise increasing cut-os for the BOLD response.

On the basis of previous studies in cognitively intact individuals, it has been difficult to decide which of two processes, phonological or lexical-semantic retrieval, characterizes best the posterior middle temporal contribution. Evidence for a(lexical-)semantic impairment in early-stage AD is abundant [9] [2] [3] [7] [11] [4] while, to our knowledge, no phonological deficits have been documented in early-stage AD until now. Our findings of impaired activity in this region in MCI therefore strongly support the lexical-semantic hypothesis.

We correlated the psychophysical changes in slope during word reading in the MCI group with fMRI responses in the posterior middle temporal gyrus. The response amplitude during the associative-semantic compared to the visuoperceptual conditions correlated strongly with the slope (parameter b) of the word identification curve (-57,-42,-6, Z=3.57, r=0.90, uncorrected P<0.001) (Fig. 3C). There was no correlation with the slope during picture naming or with any of the other parameters of the word or picture time-accuracy functions. This correlation allows us to narrow down the functional interpretation: Word-specific changes in slope of the word reading curve point to less efficient word identification. The posterior temporal correlation with the slope of word identification confirms the role of this region in lexical retrieval.

Our study provides novel insights into the earliest changes of the language system in Alzheimer's disease. In contrast with probable AD, the changes in MCI are in word-specific

rather than a-modal semantic processing structures (Fig. 4). The combination of semantic (task-dependent) and word-specific (input-modality dependent) effects in MCI fits the description of "an erosion of referential boundaries of words" [1]. Longitudinal studies will allow us to delineate the spread of alterations into input-modality independent areas as the stage of probable AD is reached [9] [2] [7] [11].

Our data have implications for the concept of 'amnestic MCI'. The exclusivity of episodic memory impairment, a main criterion for the definition of amnestic MCI, is only an apparent lack of impairment of other domains attributable to the lack of sensitivity of routine measures of semantic memory or language functioning. Word reading under time constraints and direct in vivo imaging techniques allow one to test the language system with higher sensitivity. Paradoxically, rigorous application of the criteria for amnestic MCI selects a population with associated sub-clinical language impairment. The association between amnestic MCI and a sub-clinical language impairment provide strong support for the view that amnestic MCI constitutes a prodromal phase of AD [15].

1.2.2 Left ventral occipitotemporal cortex and words

No left ventral occipitotemporal areas were more active during the associative-semantic than the visuoperceptual condition with words compared to pictures. We determined word-related responses in ventral occipitotemporal cortex in further detail [33]. The design of this event-related experiment was factorial: Input-modality (words versus pictures) and hemifield (left versus right). Stimuli were presented for 200 ms at 3 deg eccentricity. The inter-trial interval was 2260 ms. The conjunction between left- and right-hemifield word trials revealed activation of the left occipitotemporal s., at a location identical to what has been described as the visual word form area (Fig. 6A). Importantly, the conjunction between left- and right-hemifield pictures activated this area equally strongly (Fig. 6B) as well as the right-hemispheric mid-fusiform area discussed in the previous section. To the left, the amplitude of the response to words was identical to that of pictures (Fig. 6C blue and cyan versus red and magenta). The symmetrical right-hemispheric area was activated more strongly by pictures than by words, especially when the stimuli were presented contralaterally (Fig. 6D blue versus red). The effect of hemifield of presentation in the left VWFA (Fig. 6 cyan versus blue and magenta versus red) provides a strong argument in favor of a perceptual role of this region. We conclude that the occipitotemporal sulcus area has developed a special role in perceptual processing of words on top of its more ancient role in picture processing.

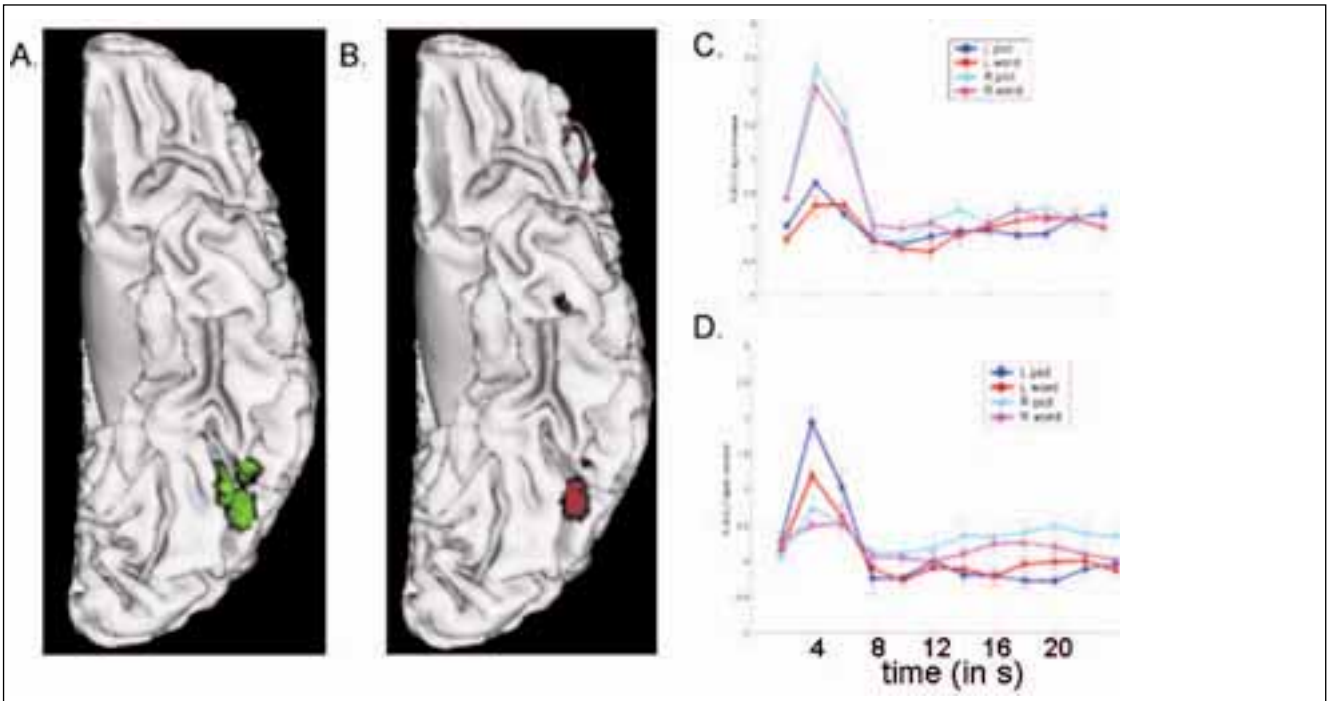

Figure 6: A. Area of conjoint activation during presentation of words in left-hemifield and during presentation of words in right-hemifield. Projection upon the ventral surface of a standard brain. Top: Anterior. Bottom: Posterior. Left-hand side: Medial side. Right-hand side: Lateral side. B. Area of conjoint activation during presentation of pictures in left-hemifield and during presentation of pictures in right-hemifield. The area entirely overlaps with that shown in A. C. Event-related response in the left visual word form area shown in A and B. X-axis: Time in s. Y axis: Adjusted fMRI response. Clearly, the amplitude of the response to words equals that of the response to pictures. Second, there is a strong effect of hemifield of presentation, with stronger responses to contralateral stimuli. Legend: Cyan: Presentation of a picture in the right hemifield. Magenta: Presentation of a word in the right hemifield. Blue: Presentation of a picture in the left hemifield. Red: Presentation of a word in the left hemifield. D. Event-related response in the symmetrical, right-sided area. Clearly, the amplitude of the response to contralaterally presented words is substantially lower than that to contralaterally presented pictures.

1.2.3 Conclusion

Our studies in normals and in patients have elucidated the function of two word-specific processing areas: The left posterior middle temporal g. and the left visual word form area. The strong hemifield dependency of the visual word form area provides strong evidence in favor of a perceptual role, related to detailed discrimination of perceptual features of printed words. The left posterior middle temporal impairment in incipient AD and its correlation with the speed of word identification in AD point to a role in two-way mapping of lexical word representations onto the distributed representation of word meaning.

A large number of behavioral studies point toward a disturbance of semantic memory rather than lexical-semantic retrieval in early- to moderate-stage AD. The longitudinal part of our study will allow us to examine how the early pathology affecting the posterior middle temporal gyrus spreads into the semantic system as the patients develop modality-independent semantic deficits on top of the word-specific lexical-semantic retrieval deficits. 8 MCI subjects have undergone a control scan with a 1-year interval and 6 are planned in the first months of 2005.

2 Input-modality independent components

2.1 Anterior temporal pole and primary progressive aphasia

2.1.1 Psychophysical study

Primary progressive aphasia is a neurodegenerative disorder that is characterized by exclusive impairment of language, leaving episodic memory intact [14]. In 12 PPA subjects, 7 patients with AD, 7 patients with MCI and 15 controls we examined the cognitive nature of the word finding deficit [31]. Six PPA patients were English-speaking and were studied at the Cognitive Neurology and Alzheimer's Disease Center, Northwestern University, Chicago, IL. Six were Dutch-speaking and were studied at the Memory Clinic, University Hospital Gasthuisberg, Leuven, Belgium. 3 women and 9 men participated, between 57 and 77 (mean 68.9, s.e. 1.9) years of age with 10 to 24 (mean 15.9, s.e. 1.1) years of education. Disease duration varied between 1.5 and 10 years. Mean Mini-Mental State Examination (MMSE) score was 26.8 (s.d. 2.5). All patients underwent an extensive neuropsychological assessment which allowed us to exclude a significant impairment in cognitive domains other than language in all subjects. Language assessment in the English-speaking patients included the Boston Naming Test (BNT) and the Western Aphasia Battery, in the Dutch-speaking patients the Dutch version of the BNT, the Aachen Aphasie Test (Dutch version) and the verbal semantic associative task of the Psycholinguistic Assessment of Language Processing in Aphasia (PALPA).

Seven PPA patients suffered from non-fluent progressive aphasia, characterized by effortful, agrammatic speech with short phrase length and distorted articulation (cases 1,2,3,7,8,9,10) and 5 from fluent progressive aphasia, characterized by effortless grammatical or paragrammatic speech with normal phrase length and normal articulation but limited content (case 4,5,6,11,12). Cases 11 and 12 could also be classified as semantic dementia patients according to the Neary et al. (1998) consensus criteria [17]. These two patients showed evidence of word comprehension deficit on clinical examination as well as on the AAT comprehension subtest and were impaired on the PALPA word association task which probes explicit associative-semantic judgments. Their performance on the pseudo-object task of the Birmingham Object Recognition Battery suggested a mild degree of associative visual agnosia, compatible with a diagnosis of semantic dementia.

For the main experimental task, *the primed picture naming task*, subjects were instructed to watch the screen during word presentation and to name the picture. In each trial a written word was presented for 250 ms in white capitals upon a black background. This was followed by a 500 ms delay (total stimulus onset asynchrony (SOA) 750 ms) and subsequent presentation of a colored picture. The written word preceding the picture could be the correct name of the picture (identity prime), a noun belonging to the same semantic subcategory (semantically related prime), a noun belonging to a different semantic category (semantically unrelated prime), or a non-word. The semantically related prime came from the same semantic subcategory as the target stimulus, the semantically unrelated prime was an animal for a manipulable object and vice versa.

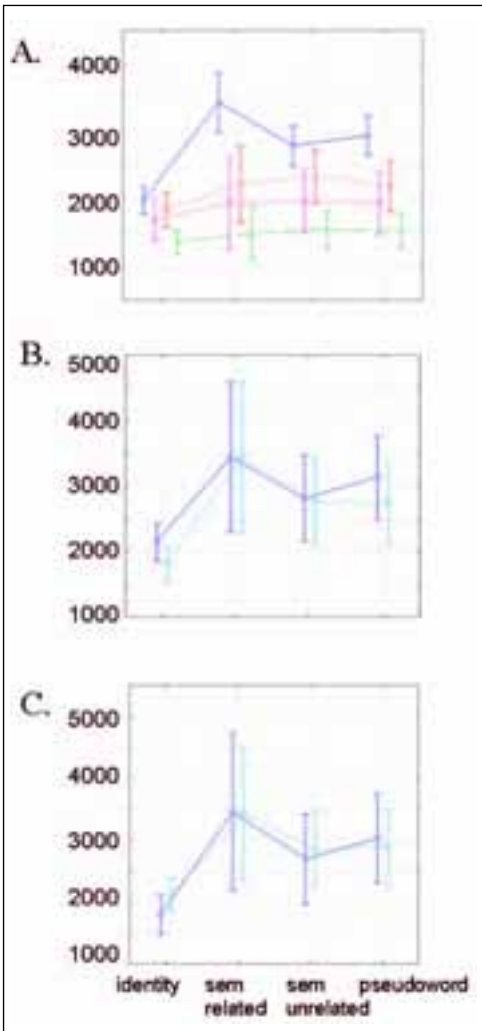

Figure 7: Primed picture naming. Reaction times (mean and s.e.) plotted as a function of prime type: Identity, semantically related prime, semantically unrelated prime and pseudoword. A. PPA patients were significantly slower following a semantically related prime in comparison to a semantically unrelated prime. The cognitively intact controls and the patients with PRAD and MCI tended to be faster following a semantically related prime in comparison to a semantically unrelated prime. Legend: Blue solid line: PPA; Red dotted line: PRAD; Magenta triangles: MCI; Green diamonds: Normal controls. B,C. PPA subgroup analysis. B. Effect of language. Dark blue solid line: Dutch-speaking; Cyan dashed line: English-speaking. C. Effect of fluency. Dark blue solid line: Fluent PPA; Cyan dashed line: Non-fluent PPA patients. Abbreviations: Sem rel: Semantically related; Sem unrel: Semantically unrelated; PPA: Primary progressive aphasia; PRAD: Clinically probable Alzheimer's disease; MCI: Mild cognitive impairment.

The main effect of group was significant ($F(3,35)$ 17.3, $P<0.00001$): Patients with PPA (mean 2787 ms, s.e. 129) or with PRAD (mean 2151 ms, s.e. 170) responded significantly more slowly than cognitively intact controls (1484 ms, s.e. 116). PPA patients also responded significantly more slowly than PRAD or MCI patients. There was no significant difference between normals and patients with MCI (mean 1884, s.e. 201) or between patients with MCI and patients with PRAD.

The main effect of prime type was also significant ($F(3,105)$ 18.7, $P<0.00001$): Subjects were significantly faster following the identity prime than in any other condition (identity prime: 1720 ms, s.e. 60; semantically related prime 2275 ms, s.e. 119; semantically unrelated 2150 ms, s.e. 93; non-word 2136 ms, s.e. 88).

Critically, the interaction between group and prime type was significant ($F(9,105)$ 7.4, $P<0.00001$) (Fig. 7A). According to planned comparisons, the reaction time difference between semantically related and unrelated primes differed significantly between PPA patients and normals ($F(1,35)$ 9.7, $P<0.005$), between PPA and PRAD ($F(1,35)$ 7.4, $P<0.05$) and between PPA and MCI ($F(1,35)$ 4.6, $P<0.05$) (Fig. 7A): PPA patients were significantly slower following a semantically related prime in comparison to a semantically unrelated prime ($P<0.000005$) (Fig.

7A), in contrast with cognitively intact controls, patients with PRAD and patients with MCI. During semantically related priming trials reaction times were significantly longer in PPA than in PRAD ($P<0.02$), MCI ($P<0.01$) or cognitively intact controls ($P<0.00001$) while reaction times during the semantically unrelated priming trials did not differ significantly between the PPA versus PRAD or MCI patients

($P<0.1$).

We performed three subgroup analyses. First, we performed a repeated-measures ANOVA with group as between-subjects factor (2 levels: Dutch- versus English-speaking PPA) and prime type as within-subjects factor (4 levels: Identity, semantically related, semantically unrelated and pseudoword) (Fig. 7B). As Fig. 7B indicates, Dutch-speaking and English-speaking PPA patients showed the same semantic interference effect ($F(3,30)$ 0.37, $P>0.7$). Second, we performed a repeated-measures ANOVA with group (2 levels: Fluent and non-fluent PPA) and prime type (Fig. 7C). As Fig. 7Cindicates, fluent and non-fluent PPA patients showed the same semantic interference effect ($F(3,30)$ 0.39, $P>0.7$). Third, we carried out a linear regression analysis with disease duration as explanatory variable and priming effect as dependent variable. There was no correlation between disease duration and priming effect ($r=0.25$, $P>0.4$).

The pathological interference seen in PPA can be understood in analogy with models for attentional search[40,41]. We postulate that PPA interferes with the discriminability of semantically related word forms during lexical retrieval even at stages when semantic processing is relatively spared. According to this scenario, the prime activates the proper semantic field and paradoxically slows the naming of semantically related probes because the correct choice becomes embedded in a field of semantically related lexical items with similar activity levels. Impaired selection among word-forms that are connected to the same semantic field can be due to degradation of a semantic field[31,42], to changes in the connections of the semantic field with word-form networks[15] or to changes within the word form networks themselves[15,43,44,45] . The actual locus of impairment may vary among PPA patients[45] .

To conclude, naming is impaired in PPA due to alteration of the process of selecting among competing word codes belonging to the same semantic field[15]. This selection deficit generalises across language groups and PPA subtypes and disease stages and constitutes a positive feature distinguishing PPA from PRAD and MCI.

2.1.2 Functional MRI study

One could argue that the absence of anterior temporal changes in amnestic MCI was due to lack of sensitivity of BOLD fMRI in this region. We can exclude this explanation. EPI images were acquired in the sagittal plane with the GeneRalized Autocalibrating Partially Parallel Acquisitions (GRAPPA) parallel imaging technique, thus maximizing sensitivity for anterior temporal activity changes [5]. Second, in a further experiment we were able to detect highly significant anterior temporal changes. We studied 13 patients with primary progressive aphasia [34]. All patients suffered from word finding difficulty in the absence of episodic memory deficit. Eight of the PPA patients also had word comprehension deficits both in the visual and

auditory modality. They participated in the same factorial fMRI experiment as the normal controls (Fig. 1A) and underwent detailed extensive neuropsychological and neurolinguistic evaluation. The test scores were subjected to a Factor analysis. The first factor consisted of a cluster of the word naming and comprehension tests as well as verbal association task. We calculated individual factor scores and correlated these scores with structural volume and fMRI responses during the associative-semantic conditions with words or pictures minus the visuoperceptual control conditions.

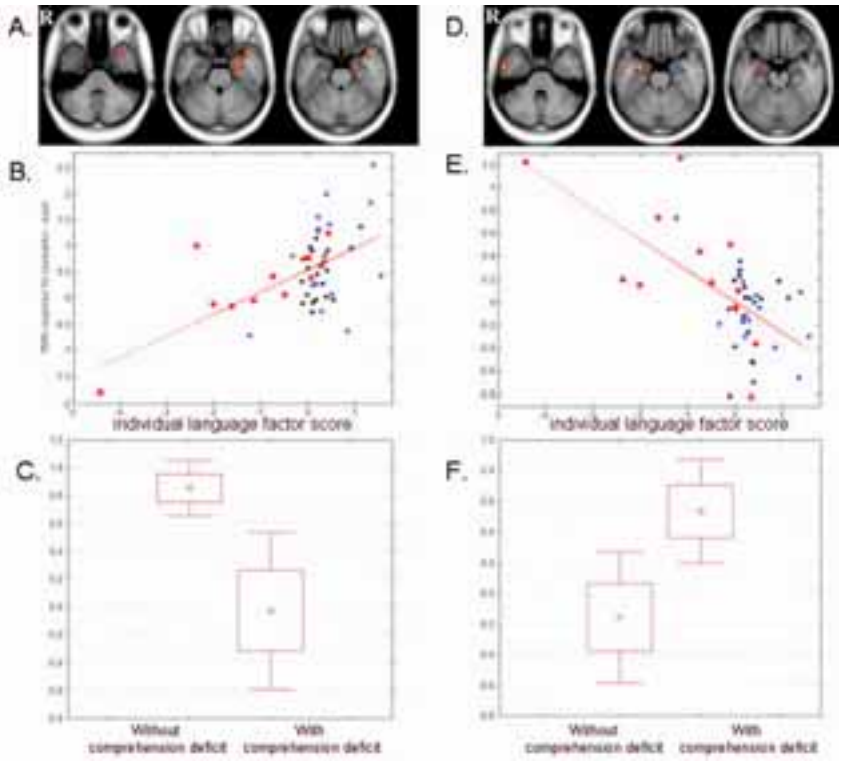

Figure 8: A. Random-effects, linear regression analysis with individual factor scores as explanatory variable (positive correlation). B. Left anterior temporal pole: Correlation between individual language factor scores (x axis) and BOLD response (y axis). Legend: Red: PPA subjects, Black: MCI subjects, Blue: Controls. C. Left anterior temporal fMRI response in two subgroups: Left: PPA subjects without comprehension deficit; Right: PPA subjects with comprehension deficit. D. Random-effects, linear regression analysis with individual factor scores as explanatory variable (negative correlation). E. Right anterior temporal pole: Correlation between individual language factor scores (x axis) and BOLD response (y axis). Legend: Red: PPA subjects, Black: MCI subjects, Blue: Controls. F. Right anterior temporal fMRI response in two subgroups: Left: PPA subjects without comprehension deficit; Right: PPA subjects with comprehension deficit.

Brain volume of one region correlated significantly with individual language competence, the left anterior temporal pole. In this same region, the BOLD response during the associative-semantic task in comparison to the visuoperceptual task correlated positively with language competence (Z=4.09, r=0.56) (Fig. 8A,B). Importantly, this was true both for words (Z=4.41, r=0.60) and for pictures (Z=4.86, r=0.63), indicating the input-modality independency of the left anterior temporal pole. A further subgroup analysis revealed that the left anterior temporal response was significantly lower in the PPA patients who had a comprehension deficit compared to those without comprehension deficit (Fig. 8C). The independence from input-modality and a role in comprehension are two criteria for attributing an associative-semantic role to this area.

In the right-sided anterior temporal pole, at a location symmetrical to the left-sided area described above, BOLD response showed a strong negative correlation with language competence (Z=4.69, r=0.62) Fig. 8D,E). Again, according to a subgroup analysis, this was only true for the PPA patients with comprehension deficits (Fig. 8F).

To conclude, pathological alterations of anterior temporal brain function can be reliably measured using fMRI. Anterior temporal integrity is required for associative-semantic processing of words and pictures, in both directions: Word finding as well as word comprehension. Third, the right-hemispheric anterior temporal pole shows an effect opposite to that seen in the left hemisphere, strongly suggesting that the right hemisphere contributes to associative-semantic processing too.

2.2 Dorsolateral prefrontal cortex and cognitive ageing

The accurate discrimination between cognitive ageing and incipient Alzheimer's disease is a pre-requisite if we want to meet the challenge of the increasing prevalence of AD in the future [27]. Within this project, we initiated a longitudinal study of 19 men and 11 women, between 50 and 90 years of age, recruited through advertisement in local newspapers. They were cognitively intact according to detailed neuropsychological and neurolinguistic assessment, both at the initial visit and after 1 year follow-up. They did not have any neurological or psychiatric history. Behavioral studies have previously demonstrated that performance of semantic memory tasks is relatively preserved during ageing, in contrast with episodic memory. We examined whether this relies on functional reorganisation of the brain system for semantic memory.

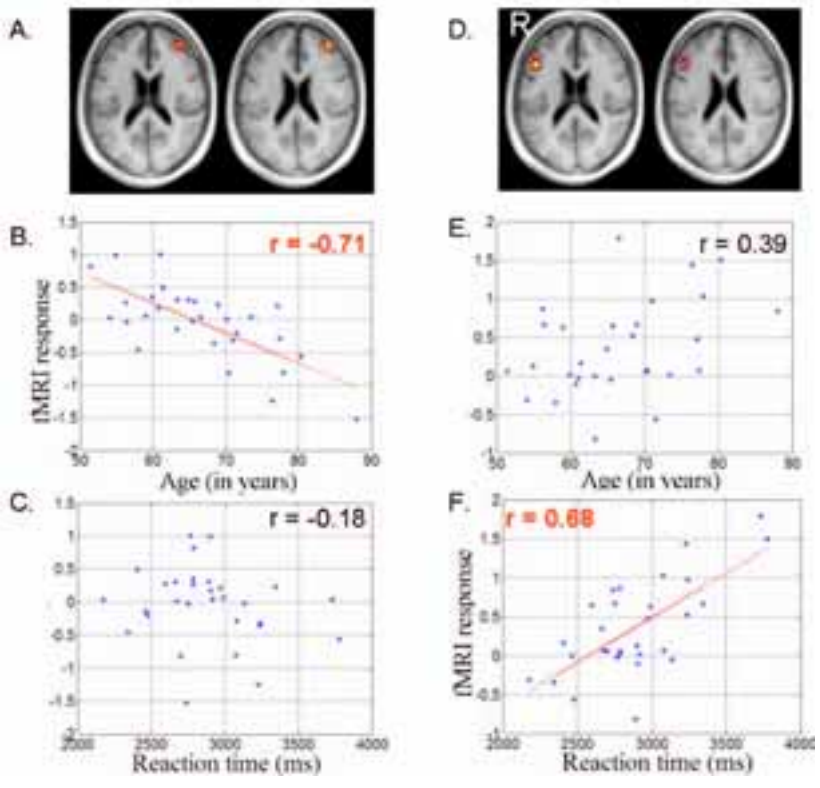

Figure 9: A,B,C. Left-sided dorsolateral prefrontal area. A. Location. B. Inverse correlation between adjusted fMRI response and age. C. Absence of correlation between fMRI response and reaction times. D,E,F. Right-sided inferior frontal sulcus. D. Location E. Absence of significant correlation with age. E. Correlation with reaction times

We conducted a random-effects, linear regression analysis with age and with reaction times as explanatory variables [29]. Age correlated significantly with the brain response in left dorsolateral middle frontal gyrus (-39,48,24, Z=4.40, r=-0.71) (Fig. 9A,B). This region lay outside of the brain system for associative semantics. The correlation was significant both when the associative semantic condition with words was compared to its control (r=-0.62) and when the same comparison was made for pictures (r=-0.58), demonstrating the input-modality independency of this region. fMRI response in this region did not show any correlation with reaction times (r=-0.18) (Fig. 9C). Reaction times correlated significantly with the brain response in the right inferior frontal sulcus (45,24,21, Z 4.21, r=0.68) (Fig. 9D,F). We carried out a stepwise hierarchical regression analysis with age, reaction times and accuracy as explanatory variables. Age but not reaction times (P0.3) or accuracies (P0.7) correlated with the left middle frontal fMRI response. Reaction times but not age or accuracies correlated positively with the right inferior frontal sulcus.

We conclude that the effect of age and reaction times is anatomically dissociable. Second, the left middle frontal g. is especially vulnerable to aging. This is in agreement with the neuronal cell loss in this area with cognitive ageing [24]. The positive correlation between right inferior frontal s. activity and reaction times leads us to the hypothesis that this region functions as a emergency generator, jumping into action when the left-hemispheric system falters.

3 Summary and conclusion

The insights provided by this project differ substantially from the a priori neuroanatomical model outlined originally at its onset (see introduction):

- According to the a priori model, the gates of entry for printed words into the semantic system were the ventral occipitotemporal junction and the inferior temporal g. (BA37). In contrast, the studies described in Section 1.2 clearly demonstrate that the left posterior middle temporal gyrus and posterior and middle third of the superior temporal sulcus fulfill this function: This region is activated during associative-semantic compared to visuoperceptual tasks with words. Dysfunction leads to impaired identification of printed words at brief stimulus durations. This region therefore is not involved in phonological retrieval as originally proposed but in mapping lexical representations onto the distributed representation of word meaning (lexical-semantic retrieval).
- According to the original model, the gate of entry for pictures into the semantic system was the lateral occipital cortex bilaterally. In contrast, the studies described in Section 1.1 clearly indicate that the right-sided mid-fusiform gyrus fullfils this function: This region is activated during associative-semantic compared to visuoperceptual tasks with pictures and dysfunction leads to impaired retrieval of perceptual features from memory. Surprisingly, mnemonic consequences of a lesion of this region cut across the classical distinctions between short-term, episodic and semantic memory.
- The original model implicated the anterior temporal poles in associative-semantic memory, regardless of input modality. Section 2.1 strongly supports this view.

- With regards to the frontal lobe, section 2.2 singles out the inferior frontal sulcus as particularly important in regulating the speed with which subjects perform the associative-semantic task with words or with pictures. This marks this region for a role in coordinating the multiple component processes that must be integrated during the associative-semantic task.

Our data also provide us with new insights into mechanisms of cognitive ageing and neurodegenerative disease:

- Ageing specifically affects the dorsolateral prefrontal cortex independently from age-related changes in processing speed.
- Amnestic MCI is associated with sub-clinical language impairment and with sub-clinical changes in the brain system for language. This supports the view that amnestic MCI is incipient AD. The earliest alterations of the language system are at the lexical-semantic level and involve the posterior temporal cortex.
- The distinction between PPA with and PPA without comprehension deficits corresponds to differential impairment of the anterior temporal pole.
- The project also brings out right-hemispheric contributions that are latent in young and healthy individuals. With aging and with neurodegeneration, activity of the right inferior frontal s. and the right anterior temporal pole, respectively, increases.

The financial support by the Queen Elisabeth Medical Foundation has allowed us to initiate a new research programme in 2001. During the course of these first 3 years, our convergent approach has allowed us to construct a neuroanatomical model of language and semantic memory and of its decline that integrates a large amount of psychophysical and fMRI data in different populations. Our next step will be to integrate cognitive and fMRI studies with in vivo imaging of pathogenetic processes, such as cholinesterase depletion [28] and amyloid deposition.

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

INTRODUCTION

The cerebral cortex is one of the most complex and important structures in our brain. In correlation with its elaborate functions, it is characterized by a huge diversity of neuronal types, each cortical neuron displaying a specific pattern of differentiation and connectivity. The mechanisms of formation of cortical networks have important implications for our understanding of pathological brain development (leading to epilepsy, mental retardation, and psychiatric diseases), and in the perspective of the rational design of replacement therapies of neurodegenerative diseases.

Over the past few years, our group has been focusing on the characterization of the ephrin/Eph family of axon guidance factors (1) during the development of connectivity of a major cortical network: the connections between the thalamus and cortex, or thalamocortical projections. We showed that members of the ephrin/Eph gene family display multiple gradients of expression in the developing thalamus and cortex, and control several important features of cortical maps (2-4).

Together with recent findings from several other groups our data provide a first framework of the genetic programme that orchestrates the development of complex neuronal networks in the neocortex (reviewed in 4,5). However many questions remain to be tackled concerning the identity of the other genes involved in this process, as well as the potential additional roles of ephrins in cortical development.

RESULTS.

1. 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. 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.

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, especially since preliminary observations suggest that these mutants display abnormal movements and ataxic gait (our unpublished observations).

In parallel to our knock-out studies, we have undertaken a complementary approach, by generating mice that display *a gain* of function of ephrin-A5, specifically in the developing cortex (6). This transgenic model, where ephrin-A5 is ectopically displayed in cortical neurons expressing its receptor EphA7, turned out to display a severe and totally unexpected phenotype: neural progenitors in these mice undergo an early and transient wave of apoptosis, resulting in a precocious depletion of the progenitor pool and a subsequent dramatic decrease of cortical size (6). Analysis of EphA7 knock-out mice revealed an exactly opposite phenotype, with an important decrease in cortical progenitor cell death resulting in an increase in forebrain size (6), similar to what was previously described in mice knock-out for several pro-apoptotic genes. Altogether these results point to ephrin/Eph signaling as a potential physiological trigger for apoptotic pathways, which raises several questions, in particular concerning the signaling pathways involved in Eph-mediated apoptosis, as well as the potential involvement of apoptosis-like pathways in ephrin-mediated guidance effects, as was recently shown for other axon guidance factors.

To address this we will use an *in vitro* model, in which treatment with soluble ephrin agonists can induce the rapid death of dissociated early cortical progenitors (6). The involvement of specific apoptotic pathways will be tested through the use of caspase inhibitors (available commercially), as well as through transfection of dominant-negative constructs of canonical apoptosis genes (Bax and Bcl2-x, caspases 3-8-9). In parallel, the effects of the same apoptosis-pathway inhibitors will be tested on ephrin-mediated guidance, using axon and cell guidance assays available in the lab. The involvement of ephrin/Eph-mediated apoptosis in other aspects of neural development and function will be explored as well, both *in vivo* (using our gain and loss of function models) and *in vitro* (using ephrin agonists and antagonists), focusing on cortical post-mitotic neurons, adult neural stem cells, as well as tumour cell lines of neural origin.

2. In vitro study of ephrins and neuronal migration in the cortex.

Recent work from a number of laboratories has demonstrated that in rodents most cortical GABAergic interneurons are generated in the ganglionic eminences (GE) in the ventral telencephalon, and subsequently migrate tangentially to the cerebral cortex through several streams of tangential migration (7). Most interneurons destined to the cerebral cortex are generated within the medial GE (MGE), while the lateral GE (LGE) essentially generates neurons destined to the basal ganglia. This dorso-ventral sorting of migrating neurons may have important pathological consequences, such as an imbalance of GABAergic innervation in the striatum and cortex (7).

Recently we have set up novel organotypic assays combined with electroporation-based transfection to dissect the molecular and cellular mechanisms that control the migration of distinct populations of neurons to specific domains of the telencephalon: in this system, GE explants overexpressing eGFP (obtained from transgenic mice or following focal electroporation) are co-cultured with organotypic slices of developing telencephalon and the migration patterns of eGFP+ cells can be monitored in real time over several days. These assays allow to recapitulate several important aspects of tangential migration, such as the dorso-ventral sorting of LGE vs MGE neurons, as well as the compartmentation of specific cell populations in the developing striatum. In parallel we have identified several candidate factors potentially involved in the guidance of forebrain migratory streams. Among these, the ephrin-B2 gene was found to be expressed selectively in the LGE (L. Passante and P.V., unpublished results), with no expression in the MGE, suggesting that it could act to restrict LGE cells from migrating tangentially to the dorsal telencephalon. This possibility is currently investigated using our *in vitro* assays: cells from LGE or MGE are transfected with bicistronic constructs (available in the lab) encoding eGFP and wild-type or truncated (dominant-negative) ephrin-B2 and their migratory behaviour assessed using confocal microscopy. In accordance with our working hypothesis, our in vitro experiments indicate that ectopic overexpression of ephrin-B2 in the MGE inhibits the migration of cortical interneurons, that remain stuck in the ventral telencephalon (our unpublished data). We are now turning to loss of function approaches, using truncated forms of ephrin-B2 that can act as dominant negative constructs. In addition, we are generating mice that display conditional loss of function of ephrin-B2 in the developing telencephalon (available in collaboration with Dr. D. Anderson's lab, Caltech) that will be analyzed both *in vitro* and *in vivo*, looking for defects in the migratory behaviour of LGE cells during development, and alteration in the balance of interneuron subtypes in the adult cortex and striatum.

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- Ephrin/Eph signaling controls brain size by regulating apoptosis of neural progenitors.
  Depaepe V, Suarez-Gonzalez N, Dufour A, Passante L, Jones K, Ledent C and Vanderhaeghen P.
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Functional magnetic resonance imaging (MRI) in awake behaving monkeys: establishing the link between human imaging and monkey electrophysiology.

One of the main objectives of our proposal was to clarify potential homologies between human and non-human primates by performing exactly the same fMRI experiments in monkeys and humans. Very good progress has been made with respect to this goal (for review see TICS, [1]). Indeed, we compared the retinotopic organization in the two species [2], the neural substrate involved in visual object processing in monkeys and humans [3,4,4], we investigated differences in motion processing between the two species [5], and compared brain regions in humans and monkeys that are involved in 3-dimensional structure from motion [6].

In another important aim, we proposed to investigate the link between haemodynamic signal changes as revealed by fMRI in the awake monkey and underlaying neuronal activity. Based upon a monkey fMRI study, we predicted that monkey area V4 contains cells selective for kinetic contours. An ongoing electrophysiological study indeed showed that, unlike neurons in areas V1, V2, and MT/V5 [7,8], a reasonable fraction (~15%) of V4 neurons respond selectively to kinetic-defined gratings and shapes (Mysore et al., 2004 Soc Neurosci abstr). To our knowledge, this is one of the first experiments where fMRI-based predictions could be tested directly in subsequent electrophysiological measurements. In another combined fMRI study in awake monkeys and humans, we compared object adaptation in shape sensitive regions of these two species. The results of these experiments are of particular importance since fMRI adaptation has been related to the decrease in the responses of macaque infero-temporal (IT) neurons after repeated stimulation (a.k.a. repetition suppression). Object adaptation in our fMRI experiments was similar in monkeys (see Figure 1) and humans (see Figure 2) (Hiromasa et al 2003 Soc Neurosci abstr). Again for this study a follow-up electrophysiological experiment is currently being carried out in which fMRI adaptation is directly being compared with repetition suppression at single cell level. A detailed comparison of fMRI adaptation and suppression effects at single unit level is of particular interest since 'fMRI' adaptation has been proposed as a way to measure neuronal selectivity in humans. The single cell results (see GSKE proposals of Drs. R. Vogels and G. Orban) indicate that the selectivity of the adapatation effect at neuronal level is narrower than the selectivity of the neuronal response (Hiromasa et al 2004 Soc Neurosci abstr). In general the latter two combined fMRI-electrophysiological studies pave the way to perform large-scale electrophysiological follow-up studies based upon fMRI results.

Finally, significant progress has been made in the last portion of my previous proposal where we aimed to investigate the functional impact of a reversible inactivation of one nodes of a functional network involved in a spatial attention task. In a first step, we trained and scanned two monkeys to perform a demanding spatial attention task in the scanner. The analysis of the results revealed attention-dependent modulation of activity in early visual areas, and a parieto-frontal network of areas, as predicted by previous human fMRI and monkey electrophysiology. (Vanduffel et al., 2004, Soc. Neurosci Abstr.). In a second step of the experiment, we successfully tested ex-vivo the possibility to perform magnet-compatible reversible inactivation experiments (using the cooling technique), which proves the feasibility of the proposed approach and that we can estimate accurately the extent of inactivated brain tissue. Currently 1 monkey is prepared and trained in a passive viewing paradigm and a

preliminary reversible-inactivation in-vivo test will be carried out soon. If these tests prove successful, we will combine the two approaches in a single subject which will enable us to measure behavioral effects of the inactivations while monitoring functional activity in all areas that are intermediate between the area being manipulated and areas involved in stimulus processing, response selection and execution.

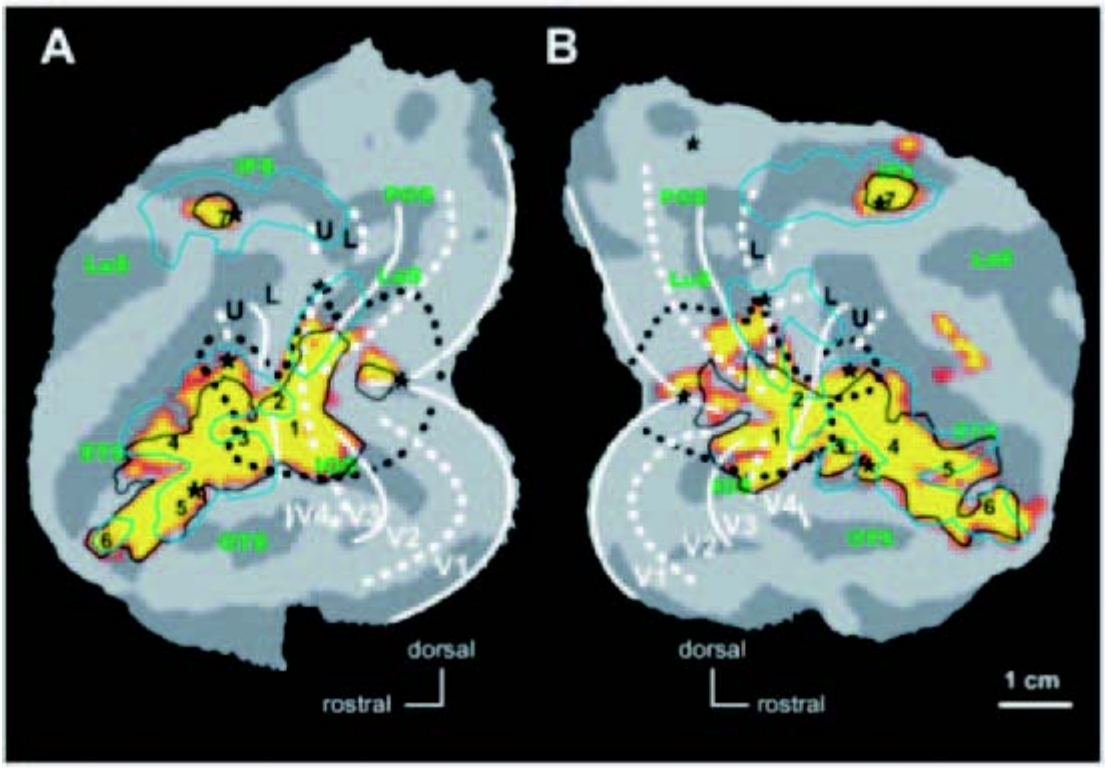

Figure 1 Adaptation sensitive regions in the monkey. SPM (group, n=3) showing voxels significant ($p<0.05$ corrected) in the subtraction '32-objects' condition minus 'identical' condition superimposed on the posterior part of flattened left (A) and right (B) hemisphere of M3. Black and light blue outlines: borders of shape sensitive regions (ie voxels significant ($p<0.05$ corrected) in the subtractions intact versus scrambled images) obtained with the small grayscale images of present experiment (black) and in the study of Denys et al 2004a (light blue). Numbers: local maxima of shape sensitive regions obtained with small grayscale stimuli (see table 1); black dotted lines and stars: central visual field (1.5 eccentricity), full and dashed white lines projection of horizontal and vertical meridians, U and L: upper and lower field (from Fize et al 2003). LaS: lateral sulcus, IPS: intraparietal sulcus, POS: parieto-occipital sulcus, LuS: lunate sulcus, IOS: inferior occipital sulcus, STS: superior temporal sulcus, OTS: occipito-temporal sulcus.

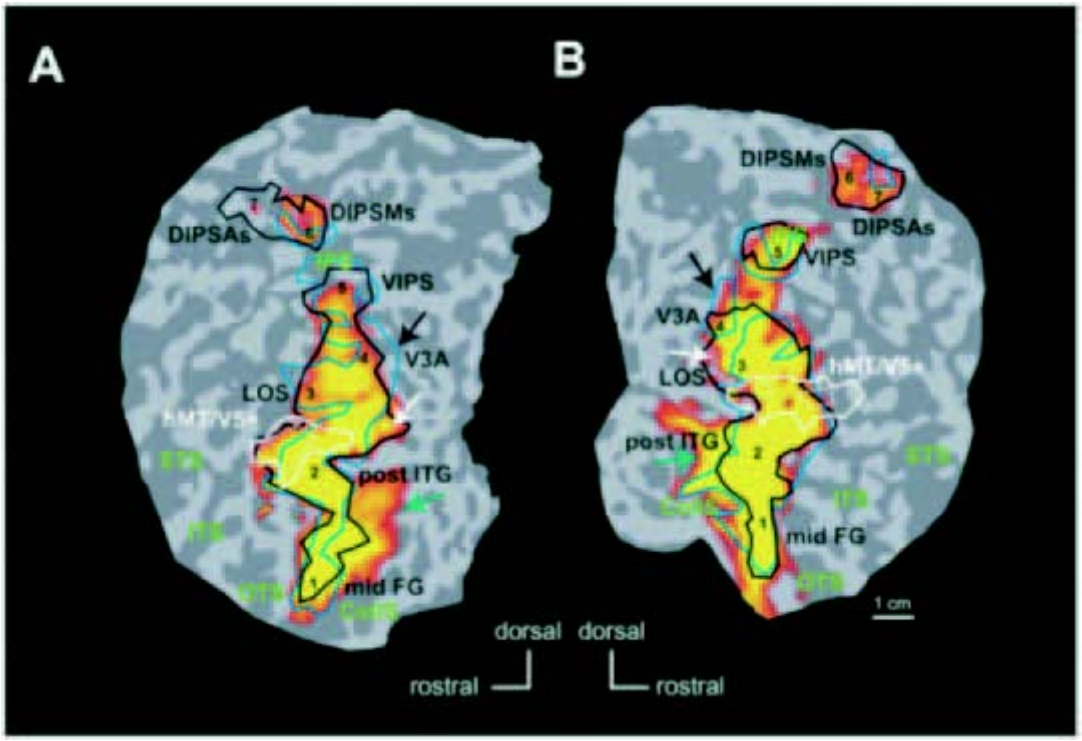

Figure 2 Adaptation sensitive regions in humans. SPM (group, n=6) showing voxels significant ($p<0.05$ corrected) in the subtraction '32-objects' condition minus 'identical' condition superimposed on the posterior part of flattened left (A) and right (B) hemisphere of the average of 9 brains. Black and light blue outlines: borders of shape sensitive regions (ie voxels significant ($p<0.05$ corrected) in the subtractions intact versus scrambled images) obtained with the small grayscale images of present experiment (black) and with the stimuli of Denys et al 2004a (light blue). Numbers: local maxima of shape sensitive regions obtained with small grayscale stimuli (see table 2). Black labels: shape sensitive regions (Denys et al 2004a): mid FG: middle fusiform gyrus (or LOa, 1), post ITG: posterior inferior temporal gyrus (or LO proper, 2), LOS: lateral occipital sulcus (3), V3A: human V3A (4), VIPS: ventral intraparietal sulcus (5), DIPSMs: dorsal intraparietal sulcus medial shape part (6), DIPSAs: dorsal intraparietal sulcus anterior shape part (7). White outlines: motion sensitive voxels (significant in subtraction motion minus stationary at $p<0.05$ corrected (full lines) or $p<10\text{-}5$ corrected (dotted lines)); IPS: intraparietal sulcus, STS: superior temporal sulcus, ITS: inferior temporal sulcus, OTS: occipito-temporal sulcus, CollS: collateral sulcus. Black and white arrows point to regions differing in shape sensitivity in the present compared to earlier experiments (Denys et al 2004a), see text; green arrows: voxels showing significant adaptation without significant shape sensitivity.

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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 how this representation is affected by learning. 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 at the single cell level the coding of cues that signal 3D shape, (2) studying the sensitivity of single IT neurons for different shape dimensions and (3) studying the responses of single IT neurons in perceptual learning task The single cell recording studies are carried out in awake rhesus monkeys.

1 Coding of 3D shape cues.

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 (Janssen et al., Science, 2000; Janssen et al., Neuron, 2000), 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 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. 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. To assess whether the texture selectivity is related to 3D shape selectivity instead of 2D texture selectivity, 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 signaled 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. The results showed that (1), as expected, the selectivity (difference between responses to concave and convex stimuli) decreased with decreasing levels of stereo-coherence for the flat texture pattern, (2) the responses at high (>40%) stereo coherences were dominated by the disparity cue, (3) the responses at <40% stereo coherences depended 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 could 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. 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 for these curved surfaces.

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 a higher saliency of the texture depth cue for planes compared to the curved surfaces that we used. 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, even higher than that observed in parietal cortex and much larger than that found for the curved surfaces in our first study. These results demonstrate clearly that in TEs there is convergence of different depth cues at the single cell level. We extended this study by measuring the responses to planes defined by different sorts of texture patterns, by comparing monocular and binocular presentations of the textures and by determining the effect of slant on the tilt tuning. Although the response of most TEs neurons was modulated by the sort of texture pattern, the tilt tuning was invariant over the different texture types and their preferred tilt was not affected by slant. This indicates that these neurons represent surface tilt in a relatively cue-invariant and abstract fashion. These results (Liu et al., J. Neuroscience, 2004) convincingly demonstrate that a ventral visual area, i.e. TEs, code for 3D texture cues.

To determine whether IT also codes for another monocular depth cue, i.e. **shading,** we used another experimental strategy that was based on the fMRI study of Georgieva et al. We determined whether IT neurons respond more strongly and/or more selectively to images of shaded 3D objects compared to images of flat, 2D shapes. The stimuli we used were similar to those used in the fMRI study on shading, i.e. "potatoes" or amoeba-like 3D shapes. We created 8 potato-like 3D shapes and each of those were illuminated from 4 different directions, yielding 32 3D shapes. For each of these 32 3D shapes we produced luminance-histogram matched 2D controls. These matched 2D controls have the same outline as the corresponding 3D shapes but have blobby like textures inside which produced a flat perceptual impression. Psychophysical ratings in humans confirmed that the 3D shapes produce a stronger depth impression than the 2D controls. A second 2D control, the scrambled control, consisted of outlines filled with pixel noise, produced by scrambling the position of the individual pixels inside the contour. We have recorded in area TEO and posterior TE of two monkeys. The TEO region we recorded from was defined based on the monkey fMRI study that showed differential activation for the 3D and 2D controls in that region.

We found a (1) a slightly (but significantly) greater population response for the 3D shapes compared to the matched 2D controls in area TEO but not TE, (2) a similar selectivity of TEO and TE neurons for 3D shapes and 2D shapes, and (3) strong effects of direction of illumination. The greater population response to 3D compared to the matched 2D shapes in TEO might underlie the stronger fMRI activation for similar 3D shapes compared to 2D controls. However, these neurons are as selective for 3D compared to 2D shapes, indicating equal coding of shading-based 3D and 2D shapes in posterior IT.

2 Shape tuning in IT cortex depends on the sort of shape dimensions.

Following up on our previous research (Vogels et al. J. Cogn. Neuroscience, 2001) on the importance of so called non-accidental shape properties for object recognition, we have recorded in 2 macaque monkeys the responses of inferior temporal neurons to a parameterized set of shapes that differ systematically along theoretically defined dimensions that define "geons". The latter are believed to be generic primitives used for categorization of objects.

Geons differ in non-accidental properties, which are shape properties that are relatively view-invariant (e.g. curved versus straight contours). Shapes belonging to the same geon-"class" differ in metric properties, which strongly depend on viewpoint (e.g. degree of curvature).We have found a greater response modulation for non-accidental shape changes compared to metric shape changes, and this for shaded objects consisting of one or of two parts and for silhouettes and line drawings. The same neurons show also a consistent and systematic tuning for metric changes. Thus, we can conclude that the representation of shape in inferior temporal cortex is versatile, supporting by virtue of the greater sensitivity for non-accidental compared to metric properties a largely view-invariant categorization of novel objects, and by virtue of the tuning to metric shape properties, discrimination of more subtle shape differences.

The above electrophysiological study (Kayaert et al., J. Neuroscience, 2003) was performed in awake, fixating monkeys. In order to determine whether monkeys show behaviorally a greater sensitivity to the non-accidental compared to metric shape changes, as humans do, we trained 3 monkeys in a temporal same-different task (2 of these monkeys were subject in the single cell study). After several months of extensive training in the same-different task, using a variety of images, we tested their matching of the shapes that were used during the single cell recordings. We found, that on average, the monkeys showed a greater sensitivity for the non-accidental compared to the metric shape changes. A psychophysical study in human subjects using the same images showed a similar behavioral bias for non-accidental shape differences. Thus, we have demonstrated the greater sensitivity for non-accidental versus metric shape changes in macaque IT neurons, macaque recognition behavior and human recognition behavior.

These results clearly suggest that the sensitivity of IT neurons does not merely reflect physical similarities but rather the perceptual image similarities, which we believe are determined by the recognition and categorization demands. This was further examined in a subsequent study (Kayaert et al., Cerebral Cortex, in press) comparing (1) IT modulations for groups of regular vs. irregular shapes, and (2) relating these neural modulations to diverse image similarity measure. This study was inspired by the psychophysical observations by Cooper and Biederman and by the proposition that object recognition favors a representation of simple, regular primitives differing in NAPs (Biederman, Psychol. Review,1987), suggesting a greater sensitivity for regular shapes differing in NAPs compared to irregular shapes. Indeed, we are more likely to spontaneously distinguish a round table from a square table than two irregular bushes and at the same time we would readily perceive that the tabletops were regular and the bushes were irregular. To what extent are these perceptual capacities reflected in the tuning of macaque inferior temporal (IT) cells? IT neurons were tested with variations of simple, symmetrical (i.e., regular) shapes, and simple and complex asymmetrical (i.e., irregular) shapes. The simple shapes could differ in NAPs. The cells were more sensitive to differences in NAPs than differences in the configuration of the convexities and concavities of irregular shapes. Multidimensional scaling showed that a population code of the neural activity can readily distinguish these groups of shapes based on perceptually salient properties such as complexity, curved vs. straight contours, and regularity, irrespective of large within-group

shape variations. Importantly, the pattern of neural modulation could not be predicted using current image-based models (i.e. pixel energies, V1-like Gabor-jet filtering and Hmax (Riesenhuber and Poggio, Nature Neuroscience, 1999)), but fitted rather well human perceptual sensitivities as measured in a shape sorting task. The representation of shape in IT thus exceeds a mere faithful representation of physical reality, by emphasizing perceptually salient features relevant for essential categorizations.

3 Perceptual learning in a backward masking paradigm and changes of IT stimulus selectivity.

Relating object recognition performance during perceptual learning 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. In humans, this perceptual learning 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 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). 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 of 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 on the responses to the shapes at short SOAs.

During the analyses of these results, we noted that in both animals responses to the masks were negligible. 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/mask differences between the two studies or reflects the extensive training was examined by recordings in two naïve, fixating monkeys. We used the same images as in the perceptual learning study. In agreement with Kovacs et al. we found mask responses in these naïve fixating monkeys. Further analysis showed that the mask responses were significantly stronger in the extensively trained than in the fixating monkeys. An additional control study showed that execution of the same-different task, without extensive training, did not decrease the mask responses. Thus this series of experiments indicate that extensive training in visual backward masking task leads to a reduction of the mask responses in those neurons that respond to the discriminanda. One interpretation of this interesting result is that before training, the population of neurons responding to the discriminanda and the population of neurons responding to the mask partially overlap, but that during training these two population become segregated.

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