Geneeskundige Stichting Koningin Elisabeth

Fondation Médicale Reine Elisabeth

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Geneeskundige Stichting Koningin Elisabeth

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Inleiding
Verslag Activiteiten van de GSKE – FMRE

Het jaar 2003 is het tweede jaar waarin de kredieten werden toegekend voor de ingezonden projecten bij de G.S.K.E.

De talrijke ploegen konden met de gebruikelijke faciliteiten, eigen aan de G.S.K.E., hun activiteiten verder zetten. De wetenschappelijke rapporten weerspiegelen de gedrevenheid van het neurowetenschappelijk onderzoek in ons land. Zij bevestigen dat de beslissing van de beheerraad, aangemoedigd door het wetenschappelijk comité, om dit specifiek thema te behouden in de G.S.K.E., de juiste keuze was.

De aanmoediging van de G.S.K.E. in de analyse van de behoeften in het kader van de problematiek van de patiënten in een persisterende vegetatieve status, heeft zijn vruchten afgeworpen. De officiële instanties en de kredietverleners hebben zeer gunstig gereageerd door een bijkomende financiering te voorzien, die waarschijnlijk in de loop van 2004 zal worden gerealiseerd. De G.S.K.E. heeft hierbij zijn imago naar een groot niet wetenschappelijk publiek ruim vergroot.

Wij danken van harte, H.K.H. Prinses Astrid voor het verderzetten van haar “werkbezoeken” aan de laboratoria. Zonder enige twijfel is dit een aanmoediging voor de onderzoeksploegen en een toenadering van de stichting naar de mensen die werken in deze instellingen.

Als woordvoerder van alle begunstigden van de G.S.K.E., is het met enthousiasme en oprechtheid dat ik mijn welgemeende dank betuig aan de leden van de raad van beheer voor hun doeltreffendheid, hun strengheid en het vertrouwen die zij in ons stellen.

Prof. Dr. Th. de Barsy

Brussel, maart 2004

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Introduction
Rapport d'Activités de la FMRE - GSKE

L'année 2003 est la seconde année d'attribution des crédits pour les projets introduits auprès de la F.M.R.E.

Les équipes, plus nombreuses, ont pu poursuivre leurs activités avec les facilités habituelles accordées par la FMRE. Les rapports scientifiques témoignent du dynamisme de la recherche en neurosciences dans notre pays et de la justesse du choix du conseil d'administration, encouragé par le comité scientifique, pour le maintien de ce thème spécifique à la FMRE.

L'encouragement apporté par la FMRE dans l'analyse des besoins pour la problématique posée par les "états végétatifs persistants" a porté ses fruits.Les instances officielles et subsidiantes ont réagit très positivement en finalisant un financement complémentaire pour ces patients, distribué probablement dans le courant de l'année 2004. La FMRE a, dans cette action, augmenté très nettement son image de marque auprès d'un public large et non scientifique.

Nous adressons tous nos sincères remerciements à S.A.R. la Princesse Astrid pour la poursuite des visites "sur le terrain"dans les laboratoires, encouragement incontestable pour les équipes de recherches et personnalisation beaucoup plus grande de la Fondation auprès des personnes qui travaillent dans ces institutions.

Etant l'interprète de tous les bénéficiaires de l'aide de la FMRE, c'est avec enthousiasme et sincérité que nous adressons nos remerciements reconnaissants aux membres du Conseil d'Administration pour leur efficacité, leur rigueur et la confiance qu'ils nous accordent.

Prof. Dr. Th. de Barsy

Bruxelles, mars 2004

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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

Actin based cell motility is essential for cell migration. During development, neural cells extend processes that are guided to their destination by short and long range repulsive or attractive guidance cues (Mueller, 1999). Formation of these processes is critically dependent on dynamic turn over of actin filaments and on transient formation of adhesive structures resembling focal contacts. Since the actin system is located in the periphery of the cell and in the filopodia of the growth cones, it is thought that the microfilament is the machinery that receives the transduced guidance information (reviewed by Suter and Forscher, 1998). An emerging picture from the last years is that the balance of actin polymerization/depolymerization is important (neither too little is good, nor too much). This balance is dictated by the activities of several actin binding proteins. The actin binding proteins studied here : **Ena/VASP-proteins, profilins, cofilins and thymosin β-members**, each modulate a different point of the actin polymerization cycle (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, see also below) necessitating to study combined effects of actin binding proteins. For instance, relevant to this project, we previously demonstrated that EVL and profilins, both actin binding proteins are also partner proteins. The simultaneous up-regulation of EVL and profilin IIa expression in brain in mouse embryos (Lanier et al., 1999, Lambrechts et al., 2000b) suggests an important role for the interaction of these proteins at this stage of neuronal development. EVL, an Ena/VASP-family member, nucleates actin polymerization *in vitro* (Lambrechts et al., 2000a) and profilins promote actin filament elongation if free polymerizing filament ends are available (if filaments are capped profilins reduce the length of filaments) (Pantaloni and Carlier, 1993; Lambrechts et al., 2000b).

The combined action of Ena/VASP proteins and profilins on actin dynamics and on neurite formation is subject of our research, as well as the effects of cofilin and thymosin β family members. With the exception of the latter family all actin binding proteins are regulated by a variety of signal transduction mechanisms. For instance EVL is regulated by Protein kinase A and possibly by interaction with n-Src kinase and FE65, profilins and cofilins are controlled by polyphosphoinositides, and cofilin in addition by LIM-kinases or other kinases.

The correct balance of actin polymerization/depolymerization appears critical for correct neuronal outgrowth. We chose actin binding proteins known or suspected from genetic or biochemical 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 cells, which display larger fan shaped growth cones than the PC-12 cells, 3) primary murine hippocampal neurons and 4) the model organism C. *elegans* (mouse will be used as a model organism in the near future). 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 (see report 2002). During 2003, a live-imaging microscopy system to monitor neurite formation over longer periods of time was set up.

C. elegans actin binding proteins in neurite formation. We devoted time to discover which of the three profilin isoforms in *C. elegans* is expressed in neurons. Although the analysis is still not complete (see below) it is clear that profilin I (PFN-1) is expressed in the neuronal ring during development (see figure in report last year, Polet et al. submitted). Similarly tetraThymosin β , of which the *Drosophila* homologue is involved in brain development (Boquet et al., 2000) is expressed in this organ (Van Troys et al., submitted). Their expression in this organ is consistent with the dynamic actin reorganization required at this stage. The knock-out of tetraThymosin β in *C. elegans* results in a lethal "dumpy " phenotype in early adults (Van Troys et al., submitted). Profilins and tetrathymosin β 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 will be used to probe tissue specific expression of these proteins, eventually enabling to monitor developing neurons. Subsequently, the promotors can also be used to drive expression of mutants or of the other profilin isoforms. This will not only allow to study rescue of the lethal PFN-1 phenotype but also to probe effects of profiling mutations on outgrowth of neurons.

We cloned the two splice variants of *unc-34* (the *C. elegans* homologue of Mena, see below) and of the *C. elegans* homologue of N-WASP (the mammalian proteins are required for correct neuronal outgrowth, see Lanier et al., 1999 and Suetsugu et al., 2002). Both proteins are potential profilin partners. Recently, using genetics, 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). The biochemical mechanism, possibly in conjunction with profilin activity, underlying this remains to be elucidated. We expressed domains of the proteins and raised antibodies against these proteins. Immunostaining will be initiated shortly. Biochemical characterization already revealed that UNC-34 forms a tetramer via its C-terminal region. Influence on actin binding dynamics in absence and presence of PFN-I, will be investigated. A PFN-1 mutant W3A (see below) aimed at disrupting the interaction with UNC-34 is being constructed and can be used for introduction in *C. elegans* (see above).

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

Mammalian actin binding proteins and neuronal outgrowth: cofilin, EVL and profilins. Cofilin is regulated by PIP_2 and by LIM-kinase. There is a clear indication that LIM-kinase inhibits cofilin activity and this reduces neurite extension (Meberg and Bamburg, 2000). The effect of PIP_2, however, was largely ignored in these studies although this phosphoinositide also inhibits cofilin activity. We engineered a PIP_2-gain of function mutant (Van Troys et al., 2000 and unpublished). Transfection of WT cofilin shows that slightly elevated cofilin levels disturb adhesion on fibronectin but not on collagen, whereas the gain of function mutant does not (Leyman et al. unpublished). As migration is coupled to adhesion/deadhesion it is interesting to investigate this mutant in PC12-cells or NG108-15.

EVL, like its family members Mena and VASP, contains an N-terminal EVH1-domain, a central proline rich region and a C-terminal EVH2 domain (Gertler et al., 1996). The proline-rich region is involved in profilin, SH3-domain and WW-domain binding (Lambrechts et al., 2000a). The EVH2-domain contains a hypothetical actin monomer binding, an actin filament binding region and a oligmerization domain, by which it form homomers and heteromers with Mena and VASP in cells (Veniere et al., unpublished). Various EVL EVH2-domain mutants aimed at disrupting its actin binding functions were constructed. The biochemical characterization of these mutants has been initiated but is hampered due to the instability of the recombinantly expressed proteins (Veniere et al., unpublished). Co-immunoprecipitation studies with GST-fusion constructs of the FE65 WW-domain and the SH3-domain of the neuronal form of Src showed that they may associate with EVL, Mena and VASP in NG108 and PC12 cells. Therefore a second set of mutants aimed at disrupting profilin and/or SH3/WW-domain binding to the proline-rich region has been constructed. This work was supported by Biacore experiments with analogous peptide mimicks and mutants some of which show dramatically reduced affinity for profilin IIa and altered stoichiometry. The long term goal here is to dissect the combined effect of EVL (or Mena) and profilin on actin dynamics, and to understand their interplay in neuronal outgrowth (see also research on profilin).

Profilins display several activities: actin binding (promotion or inhibition of actin polymerization depending on the availability of free polymerizing ends), phosphoinositide binding and interaction with proline-rich sequences 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., 2000b). These two isoforms have similar actin binding properties but have complementary affinities for the other two interaction partners (Lambrechts et al., 1997; 2000b). Genetic evidence implicates a role for profilin I in neuronal outgrowth (Lanier et al., 1999) but isoform IIa is the most abundant form in neurons. We could recently show that profilin IIa is a binding partner for SMN (survival motoneuron protein) (Sharti, Lambrechts et al., in preparation) a protein implicated in spinal muscle atrophy, a disease characterized by degeneration of motoneurons. Therefore we are addressing the role of both profilin isoforms. 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 in PIP_2 and polyproline binding) have been biochemically characterized (Lambrechts et al., 2002). We introduced these or WT profilin I in the inducible PC-12 Tet-on system. In addition, we characterized and introduced a profilin I W3A mutant defective in polyproline binding (work cited from now on is Lambrechts, Jonckheere et al. unpublished). We selected stably transfected cell lines expressing similar levels of WT and mutants allowing to compare the effects of the mutants on neurite extension. We first probed the effect on NGF/FS stimulated neurite extension after induced expression of WT profilin at three different cellular profilin I concentrations (doxycycline itself has no effect on neurite extension in the parental cell line). The number of neurites per cell decreases with increasing WT profilin I expression and thus the population of cells shifts to more cells with no or fewer neurites. Although this is consistent with the observed *in vitro* sequestering activity of high profilin concentrations, it is likely the situation in cells is more complex. Indeed, for the mutants 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 and with the parental cell line. The results are summarized in the table, representative figures are shown in the figure. Of interest is that each of the mutants, defective in one or two activities causes a different phenotype.

Table

	PC12-parental	WT profilin I	W3A profilin I	R136D profilin I	R74E profilin I
remaining activities		actin, PIP_2 polyproline	actin, PIP_2	actin (polyproline)	PIP_2 polyproline
# neurites/cell		reduced	reduced	increased	reduced
length		shorter	shorter	longer	shorter
# branches		comparable	increased	increased	reduced

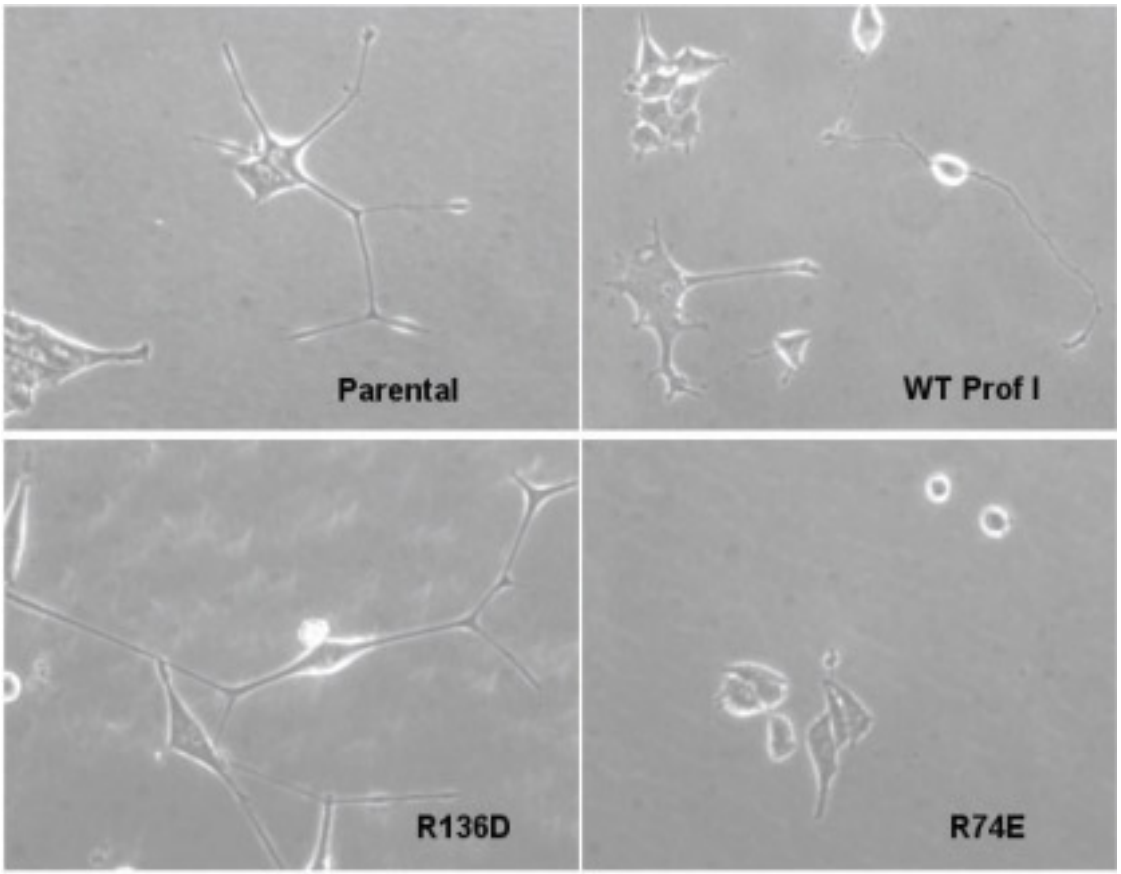

Figure: Effect of WT or mutant profilin I on outgrowth of neurites: cell lines stably expressing the various proteins at approximately 35-fold excess over the endogenous profilin I were created. Cells were stimulated with nerve growth factor and forskolin. After five days pictures were taken. Note that mutant R136D has significantly longer neurites than the parental cell line.

Cells expressing mutant R74E also have shorter and fewer neurites. Since this mutant lacks actin binding capability this may arise from dominant negative effects on, for instance, the endogenous Ena/VASP-proteins. 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 this polyphosphoinositide interaction with proline-rich actin binding protein interaction, has an important contribution in neurite outgrowth and may even be dominant over actin activity. An analogous experiment with profilin IIa shows that, similar to profilin I, it reduces the number of neurites per cell. In contrast, the W3A-profilin IIa mutant is comparable to the parental cell with respect to number of neuritis. Since this variant has Asp at position 136 it also has lower affinity for PIP_2. This results again points to the balance of the profilin interaction with PIP_2 and polyproline containing proteins. Since these experiments happened in a profilin I WT background, this will need to be combined with RNA-interference experiments. The stable profilin IIa mutant cell line will allow us to probe more specifically the effect of disruption of the profilin-Mena or -EVL interaction on neurite outgrowth.

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- Papers marked with * have been realized within the framework of GSKE
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Progress Report of the Research Group of

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

Glutamate levels and transport in cat area 17 during cortical reorganization following binocular retinal lesions.

Glutamate is known to play a crucial role in the processes of cortical reorganization after the induction of binocular central retinal lesions. 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 non-deprived peripheral area 17 together with a decreased re-uptake activity, compared to the sensory-deprived central cortex 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 in non-deprived cortex compared to sensory-deprived cortex of lesion cat and 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

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

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

γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system of mammals, plays an important role in cortical reorganization following sensory deprivation, by regulating the level of cortical inhibition and gating changes in receptive field size and synaptic efficacy. In cats it has been shown that two weeks after the induction of binocular retinal lesions, GABAergic inhibition, as determined by immunocytochemistry, is decreased in the deafferented region of area 17, whereas three months post-lesion, normal GABAergic control is restored within the cortical scotoma. In this study we used *in vivo* microdialysis to investigate the extracellular GABA concentrations one to two months post-lesion, in the sensory-deprived and remote, non-deprived region of area 17. Data were collected at those sample times and sites for which the extracellular glutamate concentrations had been determined in a previous investigation to elucidate the role of this excitatory neurotransmitter in cortical reorganization. As for glutamate, we observed significantly increased extracellular GABA concentrations in remote, non-deprived area 17, whereas in the deafferented area 17, extracellular GABA concentrations were comparable to those observed

in normal control subjects. These data suggest that one to two months post-lesion the deafferented cortex behaves like normal visual cortex, in contrast to the remote, non-deprived cortex. Notwithstanding the increase in extracellular GABA concentration with 134%, the parallel increase in glutamate concentration with 269% could give rise to a net increase in excitability in remote area 17. We therefore suggest that LTP-like mechanisms, and thereby cortical reorganization, might still be facilitated, while possible excessive hyperexcitability is balanced by the moderately increased GABAergic control.

Massie et al., Brain Research, 976:100-108.

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 central, lesion-affected region 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 in central 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 central area 17. Together, these results connect subunit-specific and time-dependent fluctuations in the composition of ionotropic glutamate receptors to adult cortical plasticity.

Van Damme et al., Cerebral cortex, in revision

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

To investigate the possible involvement of the α-subunit of the calcium/calmodulin dependent protein kinase type II (αCaMKII) in brain plasticity, we performed *in situ* hybridization and Western blotting experiments to analyze mRNA, protein and autophosphorylation levels of this multifunctional kinase primary visual cortex of cats with or without retinal lesions. No differences in the mRNA or protein levels were observed between the central, sensory-deprived and the peripheral, non-deprived regions of area 17 of 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 visually-deprived 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., Neuroscience 120:133-142.

Molecular cloning and differential expression of the cat immediate early gene *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 measure *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., Molecular Brain Research,111:198-210.

Differential display implicates cyclophilin A and MEF2A transcription factors in adult cortical plasticity

We used differential mRNA display (DDRT-PCR) to compare gene expression patterns between normal control and reorganizing visual cortex (area 17-18), 3-days after induction of central retinal lesions in adult cat. Systematic screening revealed a decrease in the mRNA encoding cyclophilin A in lesion-affected cortex. *In situ* hybridization and competitive PCR confirmed the decreased cyclophilin A mRNA levels in reorganizing cortex 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 sensory-deprived 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. Immunocytochemistry 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.

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

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

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

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

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

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

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.

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

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

The functional properties and anatomical organization of the mammalian visual cortex are immature at birth and develop gradually during the first postnatal weeks. There is a 'critical period' where the cortex is plastic and susceptible to changes in visual input. Knowledge of proteins with a high expression during this period has great importance for the understanding of activity-driven maturation of the brain. The Collapsin Response Mediator Protein 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.

Cnops et al., Eur. J. Neurosci., in revision

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

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

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

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

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

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

Combined use of Laser MicroDissection, 2-D DIGE and mass spectrometry for the identification of differential protein expression across the cortical layers of cat visual area 17

The 6 layers of the neocortex of mammals contribute differently to the functional diversity within the cortical circuitry. We compared the protein expression patterns between cortical layers

within cat primary visual area 17. Up till now, manual collection of cortical gray matter seriously hampered such a detailed laminar analysis, since the neocortex is only 2 mm thick. Application of Laser MicroDissection (LMD) allowed the separate procurement of tissue samples from the granular (IV), infra- (V-VI) and supragranular (II-III) layers of cat area 17. To this end a correct demarcation of the six cortical layers was achieved by a histochemical methylene blue staining, revealing specific cell characteristics like size and morphology. The effects of tissue preparation and staining have been investigated and were rendered compatible with subsequent protein analysis. Combination of LMD, fluorescent 2-D difference gel electrophoresis and mass spectrometry identified a first set of layer-enriched proteins in mammalian neocortex, when comparing the protein expression between supra- and infragranular cortical layers of area 17.

Neurochemical organization, architectonic subdivision and 3D-reconstruction of cat ventral lateral geniculate nucleus and monkey pregeniculate nucleus.

In contrast to the overwhelming information about the mammalian dorsal lateral geniculate nucleus (LGN), there is an astonishing lack of information concerning the neuronal organization, including neuronal morphology and neurochemical phenotype, of the ventral LGN (vLGN) in cat and the pregeniculate nucleus (PrGC) in monkey. This immunocytochemical study revealed distinguished neurochemical reactivity for neurofilament protein and seven neuronal markers to characterize the GABAergic cell population, i.e. GABA, calbindin, calretinin, parvalbumin, neuropeptide Y, enkephalin and somatostatin within cat vLGN and monkey PrGC. A characteristic neurofilament protein expression profile was apparent allowing the delineation of these subcortical nuclei into two subzones in both species. Calretinin, neuropeptide Y and enkephalin are efficient anatomical markers allowing for the parcellation of monkey PrGC in two subdivisions. In cat, GABA, parvalbumin and enkephalin immunoreactivity clearly demarcated the medial part from the lateral part of the vLGN. Based on characteristic neurochemical marker expression profiles within the vLGN and PrGC and on the position of both subcortical structures in the lateral geniculate nucleus complex, these findings suggest a well-defined part of the cat vLGN and monkey PrGC as homologue of the rodent intergeniculate leaflet. Three-dimensional reconstructions of this complete subcortical structure together with the dorsal LGN elucidate the true anatomical constellation of cat vLGN and monkey PrGC.

Van der Gucht et al. 2003, Soc. Neurosci. 29:67.8

Sweet Substitute - A software tool for in silico fragmentation of peptide-linked N-glycans

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.

Clerens et al., Proteomics, in press

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Notch signaling during vertebrate early neural development.

The Notch signaling pathway is an evolutionarily highly conserved mechanism for cell-cell communication that is important for cellular differentiation in various developmental processes (Artanavis-Tsakonas et al., 1999). In vertebrate embryos, the Notch signaling pathway has been shown to regulate both neuronal and glial differentiation in the developping nervous system, T-lymphocytes differentiation in the immune system, segmentation of the paraxial mesoderm, to name just a few examples. Despite the importance and diversity of action of Notch during vertebrate development, until now only a limited number of target genes have been identified that mediates or regulates its action during development.

Recently, we have isolated two novel genes implicated in the Notch pathway. The Xenopus Hairy-related transcription factor 1 (XHRT1) gene encodes a member of a novel subfamily of basic helix-loop-helix (bHLH) transcription factors related to the Drosophila Hairy and Enhancer-of-split (E(spl)) and the mammalian HES proteins. Compared to the other HES/E(spl) proteins, these HRT proteins are characterized by an invariant glycine instead of a proline residue in the basic region and by the substitution of the WRPW carboxy-terminal motif by a YxxW motif, followed by the TE(I/V)GAF sequence (Davis and Turner, 2001). In collaboration with the group of D. Christophe (ULB), we have shown that this gene marks in early embryos floor plate and hypochord precursor cells and that its expression in ectodermal cells is induced by Notch signaling (Pichon et al., 2002). We also analysed its DNA-binding and transcriptional activity. We found that, compared to HES1, XHRT1 binds to similar DNA sequences and that in transiently transfected 3T3 cells, XHRT1 inhibits the expression of a luciferase reporter gene under the control of a promoter containing multimerized XHRT1 consensus binding sites and thus functions as a transcriptional repressor (Pichon et al., submited). The function and mode of action of XHRT1 during midline cells development is still completely unknown.

The Xenopus XNAP gene encodes an evolutionarily conserved protein containing two repeated ankyrin motifs. We showed that the XNAP gene is strongly upregulated in response to Notch activation and that when overexpressed in embryos, it reduces the activation of Notch target genes and increases the number of primary neurons (Lamar et al., 2001; Lahaye et al., 2002) suggesting that it acts in a feedback loop as a negative regulator of Notch signaling. The mode of action of the XNAP protein as well as the mechanisms of regulation by Notch of the XNAP gene are still not determined.

We have focused this year on:

1 the study of the role of XHRT1 in Xenopus midline cell and the identification of the regions of the protein required for its activity

2 the study of the mechanisms that control XNAP gene activation by Notch

1. Functional dissection of the Xenopus Hairy-related transcription factor 1 (XHRT1) gene : importance of the Orange and C-terminal sequences for dimerization and choice of the bHLH partner.

Grafting and ablation experiments in zebrafish and chicken have shown that cells from the organizer give rise to floor plate, notochord and dorsal endoderm. As recent data in zebrafish and Xenopus have shown that Notch signaling is active in the organizer cells and plays an important role in the specification in midline cells, favoring floor plate and inhibiting the differentiation of notochord cells (Lopez et al. 2003), we wanted to know whether XHRT1 plays a role in this process. As Xhairy2b is also expressed in floor plate precursor cells and is activated earlier than XHRT1 in the organizer region (Tsuji et al., 2003), we also analysed its effects on midline cell specification. To approach their function, we overexpressed them in the embryo by microinjection of mRNAs and looked at the expression of various midline markers. We found that both XHRT1 and Xhairy2b in early gastrulating embryos inhibit the expression of the notochord markers chordin and Xbra and in contrast expands the expression in the ectoderm of the early midline marker Xnot, suggesting that they may play a role in midline cell specification.

In addition to a role in floor plate specification, Notch signaling has been also shown in chicken to be important for floor plate cells at later stage of development to maintain floor plate cell identity (Le Roux et al., 2003). We therefore overexpressed XHRT1 or Xhairy2b in the neurectoderm and looked at the expression of the N-tubulin neuronal marker. We found that both XHRT1 and Xhairy2b inhibit the expression of the N-tubulin gene. Expression of the proneural gene neurogenin and other downstream regulatory genes involved in neurogenesis (ex.: X-Myt1) has been also tested and found to be down-regulated. These results suggest that XHRT1 and Hairy2b may also play a role in late floor plate cells by preventing them to adopt a neuronal fate.

To determine the domains of the protein required for XHRT1 activity, we next generated a serie of XHRT1 deletion mutants and tested their ability in the embryo to block chordin and N-tubulin expression. From this deletion analysis, it appears that the bHLH domain is required but is not sufficient alone for XHRT1 activity. Only the XHRT1 deletion mutants that include the Orange domain or the intermediate region connecting the Orange domain to the C-terminal YRPW motif are active in the embryo.

The C-terminal YRPW motif is not involved in this repression and cannot interact with the Groucho/TLE corepressor proteins. It has been shown recently that the bHLH domain alone of HRT2 is sufficient for its transcriptional repression activity (Iso et al., 2001). Our results thus indicate that XHRT1 functions as a DNA binding dependent repressor and demonstrate that different members of the family use distinct mechanisms to repress gene expression.

To identify the regions of the protein that have intrinsic repression activity, we generated a serie of GAL4-XHRT1 fusion proteins and tested their ability to repress the expression of a reporter gene under the control of a promoter containing UAS binding sites. We found that, in

contrast to HRT2 and in accordance with the results obtained in embryos, the XHRT1 bHLH domain does not have intrinsic repression activity. One other important observation is that the Orange domain, which is crucial for the activity of the protein in the embryos and is thought to be involved in repression in the case of HES1 (Castella et al., 2000), has also weak intrinsic repression activity. These results thus further demonstrate that XHRT1, compared to other HRT factors, uses distinct mechanisms of transcriptional repression and suggest that the Orange domain is not involved in repression.

HES proteins often function as heterodimers. To identify XHRT1 bHLH partners, a yeast two hybrid screening has been performed in D. Christophes's group using the bHLHO region of XHRT1 as a bait. Using this approach, Xhairy1 (also termed HES1 in human) and Xhairy2b (c-hairy-1 in chicken) have been identified as XHRT1 heterodimeric partners. An interaction between HRT1 and c-hairy-1 has been also reported recently in chicken (Leimeister et al;, 2000). Using immunoprecipitation assays, we have confirmed those interactions. We also found that XHRT1 can homodimerize and heterodimerize with another HES factor, XHes2 (M. Soelter, unpublished), but is unable to interact with ESR8, ESR9 and Hes6r as well as with positive neurogenic bHLH factors such as neurogenin or XATH3. In gel shift assays, only heterodimers give strong retarded bands. Thus, XHRT1 can heterodimerize with a restricted number of HES family members and heterodimerization appears to be important for its activity.

In chicken, the Orange domain of HRT1 has been shown recently in a Y2H assay to enhance the interaction with c-hairy1 (hairy2b) , but it is not known whether this effect is due to a role of the Orange domain in dimerization or to an indirect effect such as protein stabilization (Leimeister et al;, 2000). To determine whether the Orange domain of XHRT1 plays a role in dimerization, we first tested in yeast two hybrid assays different XHRT1 deletion mutants for their ability to interact with XHRT1, Xhairy1 and Xhairy2b. In agreement with the results obtained in chicken, we found that XHRT1 deletion mutants containing only the bHLH domain are unable to interact and that the addition of the Orange domain, but also to a lesser extend sequences downstream of the bHLH domain, enhance the interaction. Those results have been confirmed in gel shift and coimmunoprecipitation assays. In those assays, the levels of the different deletion mutant proteins have been checked and found to be comparable.

As the sequences downstream of the bHLH domain of XHRT1 are important for the efficiency of dimerization, we wanted to know whether they are also implicated in the choice of the bHLH partner. In coimmunoprecipitation assays, we observed that ESR9, in contrast to XHRT1, cannot interact with Xhairy1 and Xhairy2b. To identify the regions of XHRT1 that are required for these interactions, we performed swapping experiments between these two proteins and tested the ability of the chimeric proteins to interact with Xhairy1. We first exchanged the sequences located C-terminal to the bHLH region. We found that a fusion protein containing the XHRT1 bHLH domain and the ESR9 C-terminal sequences cannot bind to Xhairy1 anymore while a fusion protein containing the ESR9 bHLH domain and the XHRT1 C-terminal sequences is now able to interact with it. Based on the importance of the Orange domain for dimerization, we next have created ESR9 and XHRT1 chimeric proteins where their Orange domain alone has been exchanged. We observed that the replacement of the Orange domain of XHRT1 by that of

ESR9 only slightly reduces its ability to interact with XHairy1 while the ESR9 protein with the XHRT1 Orange domain is still unable to interact. Finally,we constructed ESR9 and XHRT1 proteins were only the sequences downstream of the Orange domain have been exchanged. We found that the fusion protein containing the bHLHO part of XHRT1 and the C-terminal part of ESR9 is not able to interact anymore with Xhairy1 and that the ESR9 bHLHO protein with the XHRT1 C-terminal sequence is now able to interact with it. Together, these results indicate that the sequences downstream of the bHLH domain of XHRT1 play an essential role in dimerization. The Orange domain appears particularly important for the efficiency of the interaction while sequences downstream of it are crucial for the choice of the heterodimeric partner.

2. Study of the mechanisms that control XNAP gene activation by Notch

To determine whether the XNAP gene is a direct target of Notch and to study the mechanisms of its transcriptional activation, reporter gene assays have been performed using a luciferase reporter construct containing 4kb of upstream sequences. Using this approach we could show that the XNAP reporter construct is strongly activated by the Notch intracellular domain (Notch ICD).

To identify the cis regulatory elements of the promoter that are required for the activation by Notch, we have now generated a serie of deletions of the initial XNAP promoter reporter construct and tested them in transient transfection assays in 293T cells. The results obtained indicated that the region between -3011 and -2735 is important for the activation by Notch. Within this interval, we have identified a potential Su(H) binding site. This site appears important for XNAP gene activation as mutation of this site strongly decreases the efficiency of activation of the XNAP-luciferase reporter construct and coinjection of a dominant negative Su(H) construct (Su(H)DBM) together with Notch ICD inhibits this activation.

Further evidences that the XNAP gene is a direct target of the Su(H) mediated Notch pathway will be obtained by analysing its expression under physiological Notch stimulation through ligand binding and in Su(H)-deficient cells.

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Experimental analysis of the microcircuitry of cerebellar cortex

The cerebellum is essential for equilibrium and smooth sensorimotor coordination. Recent human brain mapping studies have also implied an important role in cognitive tasks.

The circuitry of cerebellar cortex is unique in its regularity and simplicity compared to, for example, the cerebral cortex. Many theories on the function of cerebellar cortex have been inspired by the long parallel fiber tract and by the huge convergence of inputs onto the flat Purkinje cell dendritic tree. Most such theories were first formulated more than 30 years ago. For example, Braitenberg and Atwood (1958) argued for the role of cerebellar cortex as a timing device, while Eccles (1967) described the cerebellum as a computer-like structure and proposed the "beam theory" of parallel fiber effects. Most influential was the cerebellar motor learning theory proposed by Marr (1969) and Albus (1971) which is based on long-term depression of parallel fiber synapses.

More recent experimental findings raise, however, many questions about the validity of these theories. An example is the role of the parallel fibers and their excitation of Purkinje cells. Cohen and Yarom (1998) demonstrated that mossy fiber activation does not lead to activation of Purkinje cells along a beam of parallel fibers, while our work demonstrates that at the same time Golgi cells are excited (Volny-Luraghi et al., 2002). In general, classic theories assign a minor function to the granular layer of the cerebellum. This may seem surprising as it contains more neurons than the rest of the brain. Moreover, we and others have demonstrated over the last few years several properties of the granular layer which are difficult to explain with these theories. D'Angelo et al. (1999) demonstrated that the mossy fiber to granule cell synapse undergoes synaptic plasticity under the form of long-term potentiation raising the question of how this relates to cerebellar learning.

Better theories will depend on getting a full understanding of how the local microcircuitry of cerebellar cortex works.

Electrophysiological and morphological characterization of large interneurons of the granular layer

The goal is to characterize neurochemically intracellular labeled interneurons of the granular layer of the cerebellum and to relate possible subclasses to electrophysiological properties. A review of our previous work on this subject, partially supported by the GSKE, has been published (Geurts et al. 2003).

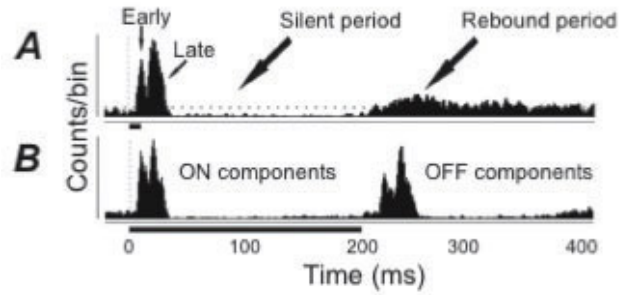
After some initial difficulties we have now established the technique of in vitro cerebellar slice preparations. Cerebellar neurons are identified using infrared differential interference contrast video microscopy (Leica DMLFS microscope, Leica microsystems Wetzlar GmbH, Germany). Whole-cell recordings are made in both voltage- and current-clamp mode by using an EPC 10 amplifier (Heka, Lamprecht/Pfalz, Germany). The path electrodes are filled with "physiological" intracellular solution that contains in addition Biocytin (0.3-0.4%). After recording, slices containing the filled cells are quickly fixed at 4°C in 0.1 M PBS containing 4% paraformaldehyde, 0.05% glutaraldehyde and 15% saturated picric acid. After 12 hr to 1 week

of fixation, biocytin is revealed using the ABC elite kit (Vector Laboratories, Burlingame, CA). With this technique, we now routinely record from Purkinje cells and consistently fill them with Biocytin (n=20). We also obtain stable recordings from neurons in the granular layer. However, these latter cells appear to be much less reliably filled with Biocityn than Purkinje cells (9 granule cells and only 1 Golgi cell). We are currently exploring the reasons for this. One possibility is that cells in the granular layer are filled but not detected. To address this, we have designed an "area protocol": whenever a cell in the granular layer (putative Golgi or granule cell) is recorded, we also fill a Purkinje cell in the same follium, close to that granular layer cell.

Recordings from large interneurons of the granular layer

An increasing number of studies have investigated the effect of stimulation parameters on neuronal response properties. We have investigated the effect of temporal characteristics of tactile stimuli, more specifically the stimulation frequency and pulse duration, on the response profile of simultaneously recorded cerebellar Golgi cells and cortical units in ketaminexylazine anaesthetized rats.

Short pulse durations (10 ms, panel A in figure) evoked in the majority of the Golgi cells both an early and a late single peak excitatory component, of trigeminal and cortical origin respectively, followed by a silent period of about 200 ms. Long pulse durations (>50 ms, 200 ms in panel B) elicited ON and OFF excitatory components in response to the stimulus onset and offset respectively, in both the cortex and the cerebellum. Neurons responded on average 7.5 ms later to the stimulus withdrawal than to the stimulus onset. Furthermore the corticopontine OFF responses in the cerebellum and OFF responses in the cortex showed congruent latency decreases and amplitude increases for longer stimulus durations (50 to 200 ms).



Decreasing the stimulus frequency similarly affected the latency and amplitude of the responses for inter-stimuli intervals shorter than 200 ms. In view of these results, we speculate that the stimulus offset is regarded as a novel input, because both paradigms resulted in similar response amplitude and latency modifications, and that Golgi cells are responsive to the interval between two stimuli rather than the frequency per se. Finally the results indicate that

a 100-200 ms time window, corresponding to the duration of the silent period in the Golgi cell responses, is of particular importance for cerebellar processing of information in the somatosensory system.

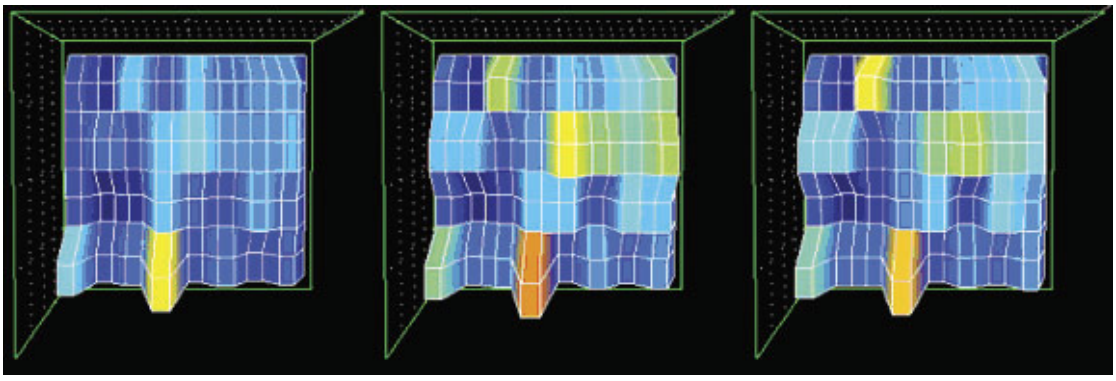
Interaction between granular layer and Purkinje cell activity in vivo

Tactile afferent pathways to the cerebellar hemispheres of the rat display a fractured somatotopical organization. The functional significance of this is not known, but it is presumed to form the basis of the integration of information from different senses or parts of the body that are used to plan and coordinate movements. In order to study the processing within and the interaction between local circuits in different receptive fields it is required to simultaneously record the activity of multiple nerve cells in different layers with high temporal and spatial resolution.

Using a new type of fork shaped micromachined silicon probe with 32 or 64 microelectrode sites (size 10 x 10 microns, input resistance 0.5-5 MOhm) distributed on 4 or 8 sharp-tipped shanks ("Vsamuel" probe, Acreo AB, Stockholm) we recorded multiple single units and multiunit activity from all layers of the cerebellar cortex (Crus I/II) simultaneously during tactile stimulation to the perioral area of the anesthetized rat.

The flow of information within and between putative receptive fields during responses to stimulation was studied by placing the probe across (sagittal) or aligned (transverse) with the parallel fibers carrying tactile information from mossy fiber afferents.

In the granular layer we found localized activity (bottom row of figure), followed by bidirectional propagation of activity in the molecular layer (upper two rows) when the 8x4 probe was placed in the transversal direction. This image is compatible with propagation along the parallel fiber beam. In the sagittal direction we observed also molecular activity but no propagation.



At present we are further analyzing the data to measure the speed of propagation. To our knowledge this is the first demonstration of parallel fiber propagation following peripheral stimulation *in vivo*.

Coding by Purkinje cells in vivo

Conventional methods of studying neuronal responses to sensory stimuli focus on measuring stimulus-related changes in spike discharge rate. Typically, spike responses, aligned to the stimulus event, are averaged across trials, yielding the stimulus-locked, time-resolved change of spike counts, as in the peri-stimulus time histogram (PSTH). More recently, there has been an increased interest in second-order measures, e.g. by quantifying the trial-by-trial variability of spike counts as measured by the Fano factor (Gabbiani and Koch, 1998). The underlying assumption in these various methods is that spike responses are realizations of a stochastic point process, with the (time-dependent) firing rate being the most relevant parameter. Here, we present first results from a new approach that assesses stimulus-related changes in the fine-temporal structure of spike responses, measured on a trial by trial basis.

We recorded the activity of Purkinje cells in the Crus II area of the cerebellar cortex in ketamine/xylazine-anesthetized rats, distinguishable by the occurrence of simple spikes (SS) and complex spikes (CS).

Here, we analyzed the SS-responses to 0.5 Hz tactile stimulation. The most common response pattern found was a short inhibition, followed by prolonged excitation, the so-called plateau response (Jaeger and Bower, 1994). Single cell response patterns were, however, quite variable from trial to trial. We found that the mean spontaneous firing rate of PCs also varied with time. Trial by trial analysis showed that response amplitude was inversely correlated with spontaneous firing rate.

Further analysis of the responses showed (1) a surprisingly large trial-by-trial variability in the duration of the initial inhibition, (2) an increased regularity of firing during the plateau response, which was masked in the stimulus-locked rate response, and (3) a systematic relation between the duration of the initial inhibition and the subsequent increase in regularity. These findings suggest that Purkinje cell responses to tactile stimuli have a much richer fine-temporal structure than hitherto thought. To what extent these various effects are systematically related to properties of the tactile stimulus is the subject of current investigation.

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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 are incorporated in the PhD thesis of Y. Jossin and are in press (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).

3. Antibodies against VLDLR and ApoER2

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. 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 is required to fulfil the reelin signal.

4. Reelin in neurological diseases

The paper describing the detection of Reelin in the CSF will be published soon (Ignatova et al, 2004, In press). 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 modification of reelin levels were detected.

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 all amniote lineages in order to assess this idea further. In order to assess whether CR cells are evolutionary homologous, we cloned parts of the p73 cDNA and carried out two coulour in situ hybridization studies in embryonic cortex of turtles, mice and crocodiles. This work showed colocalisation of reelin and p73 transcripts in neurons in the embryonic marginal zone, thus suggesting that these cells are indeed descendants from a same cell. Unfortunately, this work is not complete because we were unable thus far to clone p73 from lizards and we thus far have no data on the squamate lineage. Our efforts will now be focused on cloning the lizard p73 cDNA in order to finish this work.

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 and this analysis is almost complete. Four novel genes have been selected for further study. One of them, named clone 61, is particularly interesting as it 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 is not submitted for publication.

8. The protocadherin Celsr3

In 2002, we decided to invest some effort in studies of Celsr protocadherins, that are the orthologs of Flamingo in Drosophila. In flies, mutations in these genes affect dendritic deployment. Based on the expression patterns, we selected for more study the Celsr3 gene, which is expressed in neurons in parallel to their maturation and is developmentally regulated. In collaboration with the Neurobiology Laboratory in Namur, we inactivated Celsr3 by homologous recombination is ES cells and produced knock-out mice. These mice are available as heterozygote and the mutant will be analyzed in 2004.

9. Review on Reelin.

A review on Reelin and reelin signalling was published in Nat Rev Neurosci (Tissir et al , 2003).

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Progress Report of the Research Group of

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Collaborations

We have started up a collaboration with Prof. W.H. Evans (Dept. Biochemistry, University of Cardiff, Wales, UK) in the context of developing peptides that mimic sequences on the connexin subunits directed at interfering with connexin hemichannel function.

We also started a collaboration with the group of Prof. H. Kettenmann (Dept. Cellular Neuroscience, Max-Delbruck Center for Molecular Medicine, Buch, Berlin, Duitsland) in the context of the work on brain slices.

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. Intracellular calcium ions play a pivotal role in non-excitable cells as a messenger for *intra*cellular and *inter*cellular 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 support astrocyte-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 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[7] and to determine its involvement relative to the vesicular release pathway.

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 bloodvessels 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 might act to overcome dilution and washout of the endothelial ATP signal by the bloodflow, 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[1,6]. 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 in this context 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 are expected 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. 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. A fundamental question is whether endothelial calcium signals have effects on the transports occurring over the blood-brain barrier. We have put forward the hypothesis that astrocyte-endothelial calcium signals are instrumental in what we propose to call 'neurobarrier coupling' which, in concerted action with neurovascular and neurometabolic coupling, contributes to adapting the transport of glucose over the barrier to the local astrocytic and neuronal needs. Preliminary work suggests that certain neurotransmitters acting on endothelial calcium are 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 smooth muscle relaxation, 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 are also observed in the brain slice preparation where the tissue organization resembles the complex in vivo situation.

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Manuscripts in preparation

7. Cabooter, L. and L. Leybaert (2004). The role of intracellular calcium in ATP release through connexin-related mechanisms. In preparation.
8. Leybaert, L. (2004). Neurobarrier coupling: a partner of neurovascular and neurometabolic coupling ? In preparation.

Presentation of our results

We presented results concerning our work at the following international meetings:

- "Glio-vascular communication through ATP- and connexin-related signaling pathways", Invited talk at the FENS Meeting "Brain Damage Repair", Lisbon, Portugal, July 8-9, 2004.
- "Calcium talk at the blood-brain barrier", Invited talk at the Gordon Conference "Barriers of the Nervous System", Tilton, New Hampshire, June 28, 2004.
- "Connexin channels, connexin mimetic peptides and ATP release", Invited talk at the International Gap Junction Conference, August 23-28, 2003, St John's College, University of Cambridge, UK.
- "Communication de signaux calciques, connexines et relargage d'ATP: une étude au niveau de la barrière hémato-encephalique", Invited talk at the meeting "Intercellular communications", April 3, 2003, Université Paris-Sud, Orsay, France.

Other information

The research work performed in this project has been used by one of our collaborators, miss Katleen Braet, to obtain her PhD thesis with the title: "Calcium talk at the blood-brain barrier" (successfully defended in October 2003).

Progress Report of the Research Group of

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Part I : Pr Jean-Noël Octave (processing of the amyloid precursor protein)

Alzheimer's disease (AD) is the most frequent cause of dementia in the elderly, affecting more then 100 000 people in Belgium. The characteristic neuropathological lesions of AD are senile plaques and neurofibrillary tangles. Neurofibrillary tangles are intraneuronal lesions containing the hyperphosphorylated microtubule associated protein tau, while senile plaque are extracellular lesions containing an amyloïd core made of the amyloïd peptide Aβ that is derived from the amyloid precursor protein or APP. In less then one percent of the cases, Alzheimer's disease is inherited as an autosomal dominant disease. In these cases, gene mutations found in three different genes are responsible for the disease. The most frequent mutations have been found in the gene encoding presenilin 1 (PS1). During year 2003, the projects funded by the Fondation Médicale Reine Elisabeth allowed us to study the role of PS1 in the production of the amyloid peptide from its precursor. In collaboration with the team of Prof. J.P. Brion, we have also investigated whether mutations of PS1 could alter the microtubule network of cells expressing the microtubule associated protein tau. PS1 has also been demonstrated to be able to regulate neuronal calcium homeostasis. We have studied how a modification of calcium homeostasis is able to modify the production of intraneuronal Aβ.

1. Presenilin 1 stabilises the C-terminal fragment of the amyloid precursor protein independently of γ-secretase activity.

Cleavage of the amyloid precursor protein (APP) by β-secretase leaves the C-terminal fragment of APP, C99, anchored in the plasma membrane. C99 is subsequently processed by γ-secretase, an unusual aspartyl protease activity largely depending on presenilins (PS), generating the amyloid β-peptide (Aβ) that accumulates in the brain of patients suffering from Alzheimer's disease. If PS have been suggested to be the catalytic core of this proteolytic activity, a number of other proteins mandatory for γ-secretase function have also been discovered and the exact role of PS in γ-secretase activity remains a matter of debate, as cells devoid of PS still produce some forms of Aβ. We used an insect cell model expressing C99 to demonstrate that expression of presenilin 1 (PS1), which binds C99, not only increases Aβ production by these cells but also the intracellular levels of C99 to the same extent. Using pulse-chase experiments, we established that this is due to a markedly slowed decay of C99 in cells expressing PS1. In a CHO model physiologically producing C99 from full-length human APP, similar results were observed. Finally, we show that a functional inhibitor of γ-secretase does not alter the ability of PS1 to increase intracellular C99 levels. This suggests that binding of PS1 to C99 does not necessarily lead to its immediate cleavage by γ-secretase, which could be a spatio-temporally regulated or an induced event, and brings biochemical evidence to the existence of a substrate docking site on PS1.

2. Mutant presenilin 1 proteins induce cell death and reduce tau-dependent processes outgrowth.

The expression of familial Alzheimer's disease mutants of presenilin-1 (PS1) proteins has been observed to induce cell death in cellular systems. To investigate how this phenomenon might be associated to alterations of the microtubule network, we have studied the effect of wild-type and mutant (C263R, P264L and delta9) PS1 proteins expression on the formation of microtubule-dependent processes outgrowth and the association of PS1 to the insoluble cytoskeletal fraction in a cell line expressing the tau microtubule-associated protein. Expression of wild-type and mutant PS1 was associated with increased cell death, most marked for the P264L and delta9 mutants. The three PS1 mutants induced a significant reduction of the length of cell processes. These effects were not associated to a change in tau phosphorylation. However, the mutant PS1 proteins increased the proportion of insoluble tau in the cytoskeletal fraction and they were concentrated in the same fraction. These results suggest that PS1 proteins interact with the microtubule network, affect its organization and that this phenomenon, more marked for the PS1 mutants, might play a role in the cell dysfunction induced by mutant PS1 proteins.

3. Intraneuronal amyloid-β1-42 production triggered by sustained increase of cytosolic calcium concentration induces neuronal death.

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 β-amyloid peptide (Aβ). Aβ is produced by the cleavage of the amyloid precursor protein (APP). Since impairment of neuronal calcium signaling has been causally implicated in aging and AD, we have investigated the influence of an influx of extracellular calcium on the metabolism of human APP in rat cortical neurons. A high cytosolic calcium concentration, induced by neuronal depolarization, inhibits the α-secretase cleavage of APP and triggers the accumulation of intraneuronal C-terminal fragments produced by the β-cleavage of the protein (CTFβ). Increase in cytosolic calcium concentration specifically induces the production of large amounts of intraneuronal Aβ1-42, 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 Aβ, 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 Aβ1-42 from human APP. This accumulation of intraneuronal Aβ1-42 induces neuronal death, which is prevented by a functional γ-secretase inhibitor.

Part II : Dr Emmanuel Hermans (regulation of neurotransmission in the central nervous system)

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

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 principal research theme of the neuropharmacology group in the laboratoire de Pharmacologie Expérimentale of the Université catholique de Louvain concerns the study of the mechanisms of the regulation of receptors and other pharmacological targets involved in nervous transmission (in particular cell membrane transporters). In this respect, the aim of this research funded by the Fondation Médicale Reine Elisabeth is to characterise the fundamental mechanisms involved in the regulation of glutamate transporters in diverse models reflecting the physiology of glial cells. As indicated below, during year 2003, the regulation of glutamate transport (and glutamate transporter expression) has been studied in models of primary cultured astrocytes and in mesenchymal stem cells exposed to growth factors favouring their differentiation into glial-like cells.

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

Glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system (CNS). After release, glutamate is quickly removed from the synaptic cleft by a group of Na^+-dependent glutamate transporters among which GLT-1 and GLAST are predominantly expressed on astrocytes. There is considerable evidence that the activity of glial glutamate transporters can be dynamically regulated. However, little is known about the physiological stimuli that contribute to such process. As astrocytes were shown to express group I metabotropic glutamate receptors (mGluR1 and mGluR5), the present study was aimed at evaluating their possible involvement in the regulation of the glutamate transporters. We elaborated a model of primary culture of rat cortical astrocytes (approximately 90% of positive GFAP cells) in which the functional expression of glutamate transporters and receptors was monitored by fluorescence imaging using the Na^+ and Ca^{2+} sensitive dyes (SBFI and Fura2), respectively. In optimal culture conditions, the vast majority of cells were found to express glutamate transporters (mainly GLAST) and at least 60% responded to the group I mGluR agonist (S)-3,5- dihydroxyphenylglycine (DHPG). The modulation of glutamate transporter activity was evaluated by measuring [3H]-aspartate uptake in cells previously stimulated with DHPG (50 lM). Our results show that exposure to this agonist significantly enhanced the transporter activity (up to 30%). This effect was only observed after brief treatment (15 s) and was inhibited by a highly selective mGluR5 antagonist, 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP). In contrast, prolonged treatments (up to 48 h) had only modest effect on the activity of glial glutamate transporters. In conclusion, these results suggest that mGluR5 may contribute to the control of the glutamate transporters activity in astrocytes. Further studies are in progress to assess the physiological relevance and to elucidate the biochemical mechanism involved in such regulation.

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

Adult bone marrow constitutes a well documented source of mesenchymal stem cells. These are multipotent cells that can proliferate and 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, both in vivo and in vitro. 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 bone marrow 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, an intermediate filament protein expressed by neural stem cells. Simultaneously, a robust induction of the high affinity glutamate transporter GLT-1 (and to a lower extend GLAST) expression was detected by RT-PCR and immunocytochemistry. The expression of glutamate transporters was correlated with a dramatic 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.

Publications in 2003 in relationship with research supported by the FMRE.

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  Submitted.

Abstracts

- de Hemptinne I., Maloteaux J.M., Hermans E. (2003)
  Caracterisation of glutamate uptake in bone-marrow mesenchymal stem cells after in vitro differentiation. Belact meeting Cell Therapy: From stem cells to gene therapy (Jette, Belgium)
- Vermeiren C., Najimi M., Maloteaux J.M., Hermans E. (2003)
  Metabotropic glutamate receptor mediated regulation of glutamate uptake in primary culture of astrocytes. Belgian Society Of Fundamental And Clinical Physiology And Pharmocology (Gent, Belgium)

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Introduction

During this year, we focused on setting up the MR suite, in order to make possible the fMRI studies in cognitive neurosciences in the best experimental conditions.

The MR suite is now nearly complete, after the successful installation of: (1)The computer-assisted presentation of visual stimuli (The luminance and colorimetry of the projector have been calibrated); (2) The computer-assisted presentation of auditory stimuli through high-quality electrostatic hearphones; (3) The computer-assisted synchronization between the stimulus presentation (auditory or visual) and the scanning sequence (precision < 5 ms); (4) The recording of subject's key presses (precision < 5 ms); (5) The recording of subject's eye movements and pupil size.A EEG system is to be installed in spring 2004. This will allow us to proceed to our main interest, i.e. studies of states of modified consciousness (sleep, unconscious patients). These studies require considerable technological expertise and development and will progressively be designed in the next few years.At the beginning of January 2004, three studies have been completed during daytime. Their analysis is under way.

Part One. States of altered consciousness

Study of the non image forming system

Due to our interest in the relationships between sleep and wakefulness, we first considered exposure to light among the different factors regulating sleep. Light information is interpreted by two different systems in the mammalian brain. The classical visual system generates images of the external world, using the rods and cones as its retinal photoreceptors. The neural pathways involved in image formation have been described in great detail. Another system, which does not form images but detects changes in irradiance, has been characterized recently. This non image forming (NIF) system has been shown to exert powerful effects on physiology and behavior including long-term effects such as synchronization of the circadian clock, and more immediate effects such as suppression of melatonin synthesis. In both rodents and humans, evidence is accumulating that irradiance information is received by a novel photoreceptor as well as the rods and cones. 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 (SCN), the master circadian pacemaker.

In humans, alertness deteriorates when wakefulness is extended into the biological night and this deterioration can be countered by light exposure in a dose-dependent manner. The cerebral correlates of this NIF response to light have not yet been characterized. In collaboration with Professor Dijk (University of Guilford, Surrey, UK), a renowned specialist in circadian regulation of sleep/waking cycles, we designed a study to identify correlates of this NIF response in the absence of the confounding influence of the image forming system. Usin positron emission tomography, we assessed regional cerebral blood flow (rCBF) twelve times at 20-minute intervals between 0:30 and 4:30 am in darkness following exposure to light of varying duration in a group of 18 normal participants (figure 1). During the scans, the attention set was kept constant by asking the subjects to count deviant sounds in an auditory oddball

paradigm while fixating a red diode which is unlikely to elicit any NIF response.

The light exposure regime (Fig 1A) elicited the well-known and robust suppression of melatonin followed by a subsequent increase in darkness (figure 1B). Indeed, statistical analysis revealed that melatonin concentration decreased during light exposure and increased during darkness [$F(1) = 11.83$, $p = 0.0055$]. This regime was also associated with a significant modulation by light of the decline of alertness that occurs in the course of the biological night (figure 1C). Alertness of the subjects, assessed by the Karolinska Sleepiness Scale, decreased along the night [$F(2) = 15.35$, $p = 0.00005$]. Moreover, this decrease was significantly attenuated by light exposure [$F(3) = 5.36$, $p = 0.0037$].

In contrast, no modification was noted for the performance on the auditory task, which was near maximal under all conditions (0 - 1 error/scan). Likewise, the P300 amplitude and latency did not vary significantly along the night or with light exposure condition.

The main analysis of PET data identified the brain areas where the rCBF was significantly modulated by the duration of the light exposure immediately preceding each scan. Several cortical areas were more active in proportion to the previous light exposure: the striate cortex, the right intraparietal sulcus (IPS) and, bilaterally, an extrastriate area (table 1, figure 2). Psychophysiological interactions showed that the linear relationship between the regional activity in IPS and in the striate cortex is modulated by the duration of the previous light exposure. The present results show that light exposure have profound effects on subsequent cortical activity. The regional distribution of the effects suggests the light-induced recruitment of a set of cortical areas involved in attention.

In contrast, a significant decrease in rCBF was found in the suprachiasmatic region (peak voxel = [8 0 -10]; $Z = 3.27$; $pSVC = 0.02$; figure 3). In rodents, the firing rate of SCN neurons typically increases during light exposure but shows a substantial and prolonged (i.e., several minutes) undershoot when the light is switched off [(24), their figure 5]. The responsiveness of SCN neurons is maximal during night time. In the present study, most of the scans started near immediately after the light was switched off (figure 1). A significant decrease in rCBF at switch off in the suprachiasmatic region is thus consistent with the animal data (figure 3). The spatial resolution of PET scanning does not allow us to specify the nuclei included in the deactivated area. We surmise that the latter correspond to the SCN and other hypothalamic structures involved in NIF responses in rodents, such as the subparaventricular zone or the ventro-lateral preoptic area.

These results are submitted for publication.

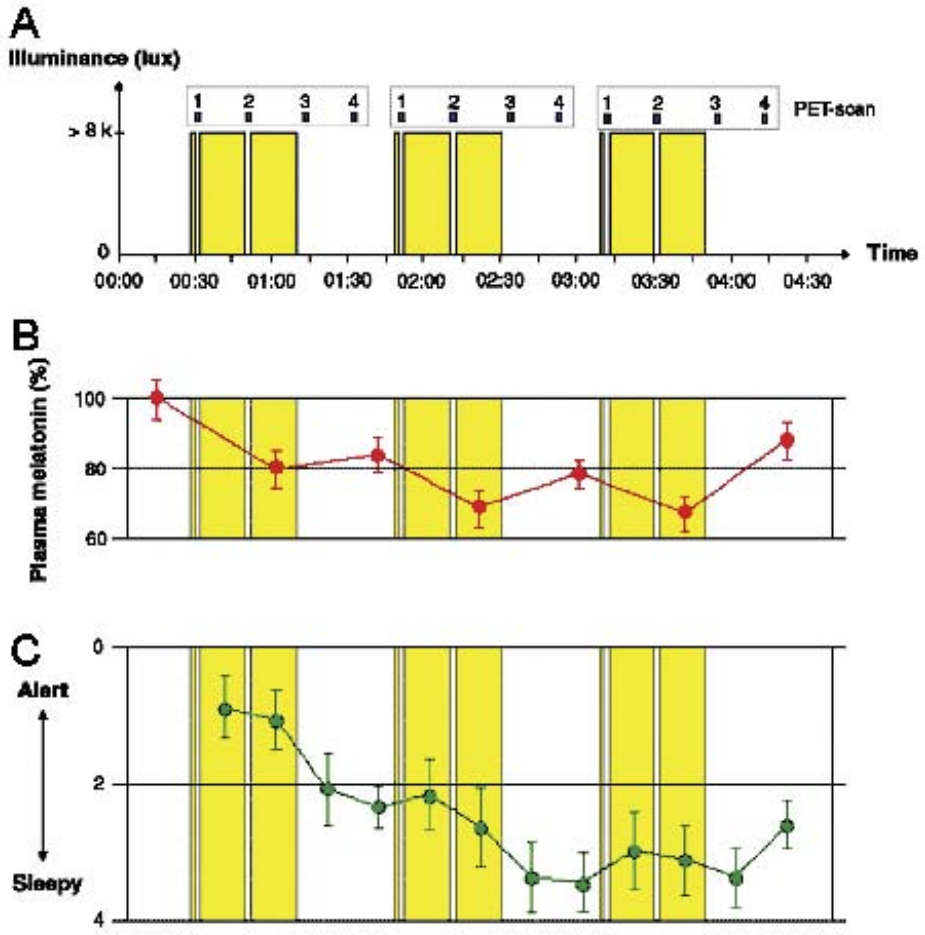

Figure 1. Experimental design (top) and neuroendocrine (middle) and behavioral results (bottom). A. The 3 blocks of 4 scans with their respective light exposure. B. Plasma melatonin samples were obtained after exposure to darkness (4 samples) or light (3 samples) and were expressed relative to the last sample obtained before scanning. C. Alertness of the subjects as assessed by the Karolinska Sleepiness Scale, expressed as changes from the averaged score of the baseline period (arbitrary units).

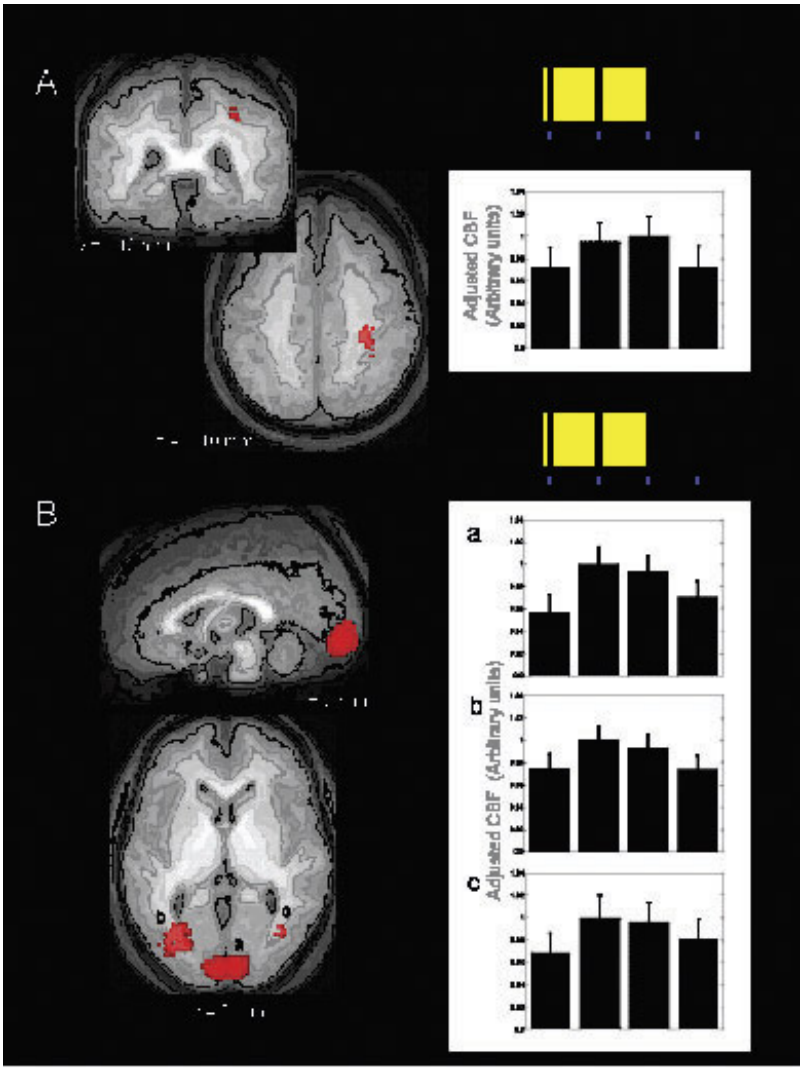

Figure 2. Cortical areas where the rCBF is significantly increased in proportion to the duration of the previous exposure to light. Left panels : functional data displayed at $p < 0.05$ (voxel level), superimposed on the mean normalized MR scan. The relevant coordinates are in mm within the stereotactic space. Right panel : plot of the adjusted rCBF at the coordinates displayed in table 1 (errobars = SEM) for the 4 scans of the blocks. For display, the adjusted rCBF were normalized to the maximum rCBF. A. The right intraparietal sulcus (IPS, frontal and transverse views). The activated area lies in the depth of the anterior part of the IPS. B. The striate cortex and extrastriate areas (sagittal and transverse views). a. striate cortex; b. left extrastriate cortex; c.right extrastriate cortex

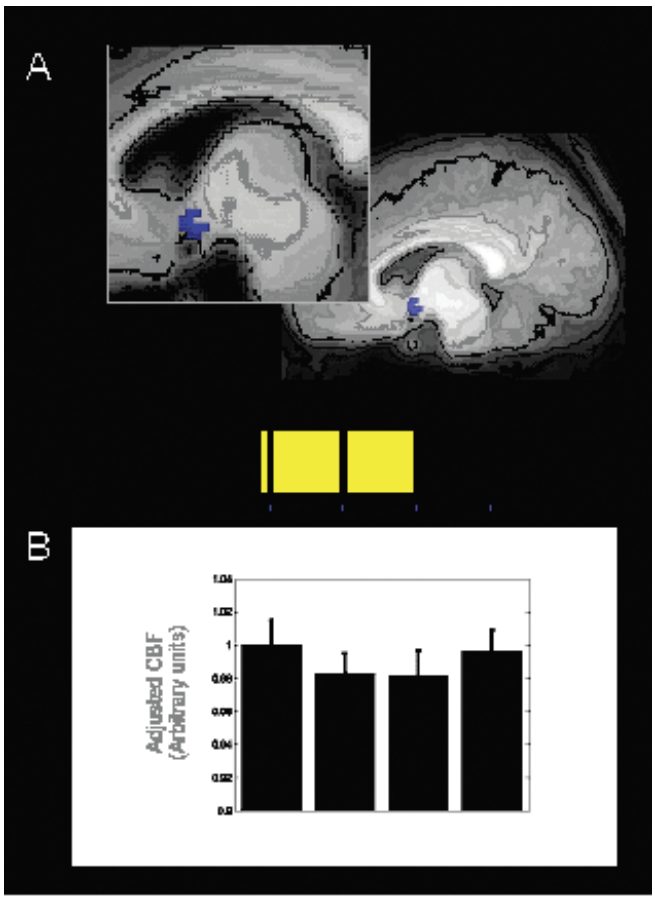

Figure 3. Suprachiasmatic area where the rCBF is significantly decreased in proportion to the duration of the previous exposure to light. A. Functional data displayed at $p < 0.001$ (uncorrected), superimposed on a parasagittal view of the mean normalized MR scan (x coordinate : 2 mm). Inset : blow up of the hypothalamic area. B. Adjusted rCBF (errobars = SEM) for the 4 scans of the blocks at [8 0 -10] mm. For display, the adjusted rCBF were normalized to the maximum rCBF.

Neural correlates of sigma and delta activity during human sleep

We also pursued our investigation of the cerebral correlates of neurophysiological mechanisms active during human sleep. Using regression techniques, we previously showed that the activity of the lateral geniculate nuclei and of the striate cortex is linked to the density of spontaneous eye movements during paradoxical sleep, but not during wakefulness, giving support to the hypothesis that PGO-like mechanisms are present in human paradoxical sleep likewise in animals (Peigneux et al. 2001, Neuroimage 14(3): 701-8). We have now investigated the neural correlates of sigma and delta activity, which characterize slow wave sleep. Sigma and delta activity in humans were previously investigated in relation to regional cerebral blood flow (rCBF) changes using $H_2{}^{15}O$ PET (Hofle et al. 1997, The Journal of Neuroscience 17(12): 4800-8). The authors found a significant negative correlation between delta activity and rCBF in the thalamus, brainstem reticular formation, cerebellum, anterior cingulate, and orbitofrontal cortex. They also found a significant negative covariation between spindle-related (sigma) activity and the residual rCBF in the medial thalamus. Although interesting, the results deserved replication and better control. Indeed, the study was conducted on a reduced data sample of six sleep-deprived subjects (total 32 scans), and the relation between thalamic activity and sigma activity was only found after the effect of delta was removed from the analysis. Here, we re-used data from prior sleep studies in which we scanned a larger population of 23 non sleep-deprived subjects (161 scans obtained during wakefulness [n = 46], stage 2 sleep [n = 50], and SWS [n = 65]). Using delta and sigma power as independent parameters, we found a significant decrease in rCBF as a function of sigma power limited to the dorsal thalamus bilaterally (local maximum on Talairach coordinates x = -6, y = -22, z = 14; t = 4.33) (Figure 4). The negative correlation of sigma activity with rCBF in thalamus can be understood as reflecting a hyperpolarization-promoted mechanism, in accordance with previous models of thalamocortical networks producing spindles under the influence of inhibitory post-synaptic potentials (IPSPs), notably driven by specific patterns of afferents arising from the thalamic reticular nuclei. Likewise, the largest negative covariation of delta power with rCBF was found in the thalamus. It is known that some of the delta waves, called clock-like delta waves, are generated in the thalamus, following a process similar to spindles but at a higher level of hyperpolarization (Steriade, 1999, Steriade and Amzica, 1998). In addition, negative correlations between delta power and rCBF were found in basal forebrain, lenticular nuclei, prefrontal regions including medial frontal gyrus and orbital cortex, inferior parietal lobule, anterior part of the insula, anterior and posterior cingulate gyrus (Figure 5a). These areas include potentially permissive (basal forebrain, anterior cingulate cortex, insula) as well as executive (thalamocortical loop and maybe basal ganglia) structures of NREM sleep. Interestingly, the map of brain regions in which delta power correlated negatively with rCBF closely resemble the previously published (Maquet et al., 1997) map of brain regions in which rCBF significantly decreases during SWS compared as compared to REM and wakefulness (Figure 5b). Theses similarities underline the notion of delta power as an accurate physiological parameter of sleep depth.

The study has been reported in international conferences and the manuscript gathering these results is submitted: T.T. Dang-Vu, P. Maquet, S. Laureys, C. Degueldre, F. Perrin, C. Philips and P. Peigneux, *Cerebral correlates of spindles and delta activity during human sleep-wake cycle*.

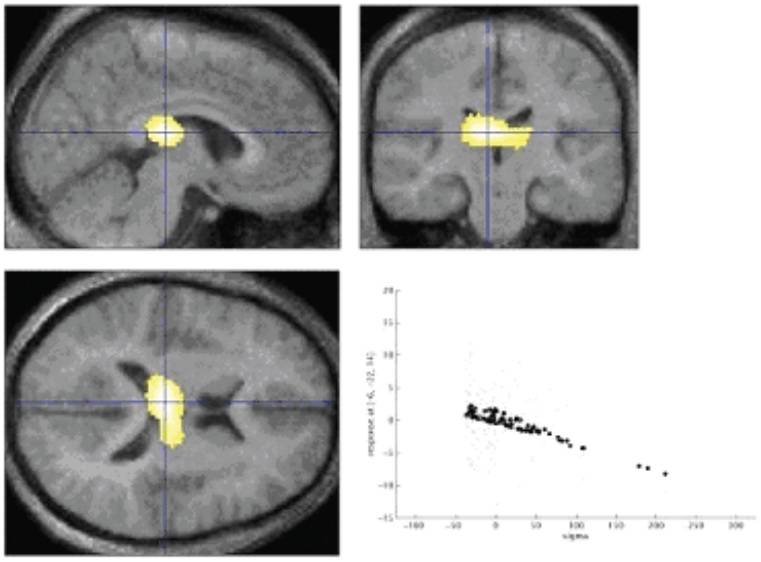

Figure 4: Negative correlation between CBF and relative sigma power in the dorsal thalamus (peak coordinate: x = -6 mm, y = -22 mm, z = 14 mm; Talairach and Tournoux, 1988), throughout wakefulness, stage 2 and slow wave sleep.

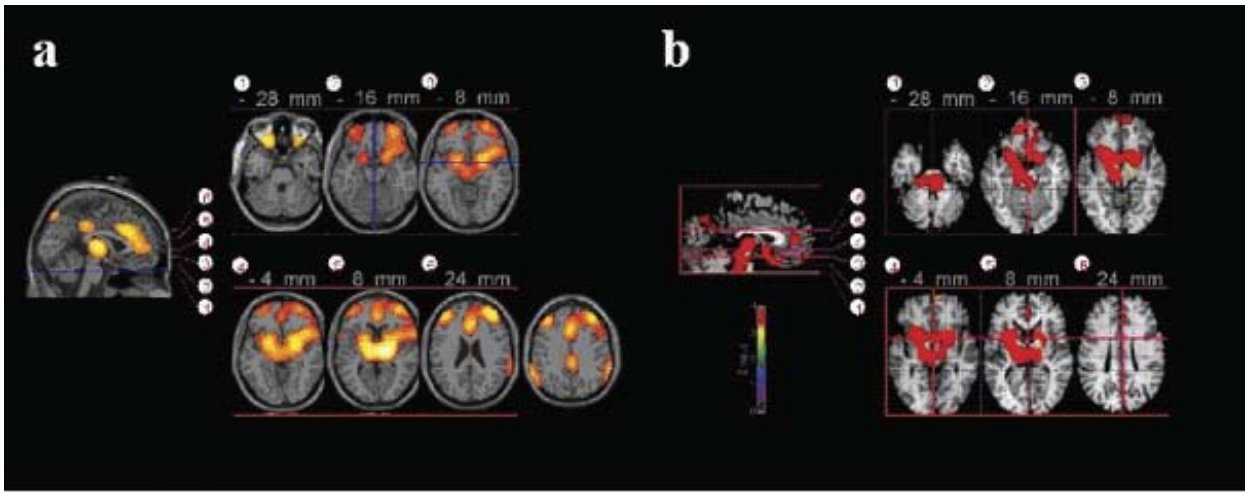

Figure 5: Statistical maps showing the brain areas in which (a) rCBF negatively correlates with relative delta power throughout wakefulness, stage 2 and slow wave sleep [left panel], and (b) in which regional CBF decreases during SWS as compared to wakefulness and REM sleep [right panel; from Maquet et al. 1997, The Journal of Neuroscience 17(8): 2807-12]. Note the striking similarity of the regional distribution of activated areas between the two maps.

Learning-dependent reactivations of brain activity during human sleep

One of our main interest is to study the relationships between sleep, brain plasticity and memory consolidation. The protocols of our related PET experiments have been already described with complete or preliminary results in previous activity reports. At this point, the definite results from this set of PET sleep studies are as follows.

First, we have previously shown that several brain areas are activated both during sequence learning at wake and during subsequent rapid-eye-movements (REM) sleep (Maquet et al., Nature Neuroscience, 831-836, 2000), suggesting that REM sleep participates in the reprocessing of recent memory traces in humans. We have further shown that regional cerebral reactivation during post-training REM sleep is not merely related to the acquisition of basic visuomotor skills during prior practice of the serial reaction time task, but rather to the implicit acquisition of the probabilistic rules that defined stimulus sequences. Moreover, functional connections between the reactivated cuneus and the striatum -- the latter being critical for implicit sequence learning -- are reinforced during REM sleep after practice on a probabilistic rather than on a random sequence of stimuli (Figure 6).

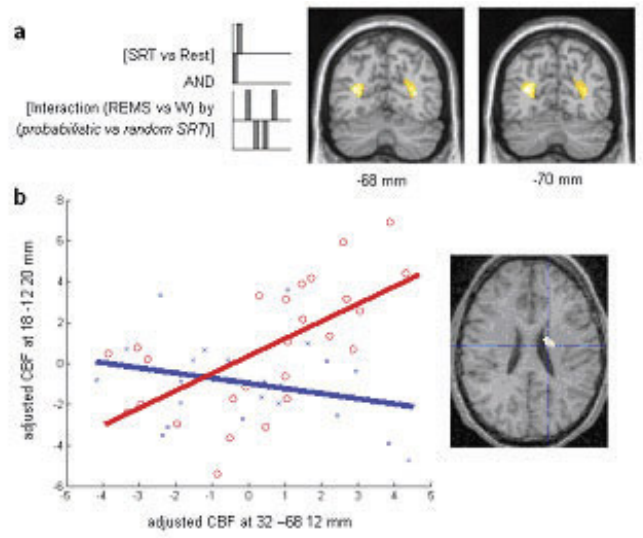

Figure 6 (a) Sequence-dependent regional cerebral reactivations during post-training REM sleep. Statistical parametric maps of the bilateral cuneus, that both activated during SRT practice (versus rest) and activated more during REM sleep (versus wakefulness; W) in Probabilistic than Random group, superimposed on the coronal section of a subject's normalized MRI at 68 and 70 mm behind the anterior commissure. Grey bars displayed on the left side of the images illustrates the subtraction (top) and interaction (bottom) contrasts entered in the SPM99 conjunction analysis. (b) Sequence-dependent increase of functional connectivity during post-training REM sleep. Plot of the regression of centered rCBF in the right cuneus (32 -68 12 mm) and right caudate nucleus (18 -12 20 mm) during post-training REM sleep in subjects trained to the probabilistic SRT task (red circles) and subjects trained to the random SRT task (blue crosses). A similar regression is observed between cuneus and caudate nucleus in the left hemisphere. The inset on the right side show the activation of the right caudate nucleus, superimposed on the transverse section of a subject's normalized MRI.

Our results therefore support the hypothesis that REM sleep is deeply involved in the reprocessing and optimization of the high-order information contained in the material to be learned. In addition, we have showed that the level of acquisition of probabilistic rules attained prior to sleep is correlated to the increase in regional cerebral blood flow during subsequent REM sleep. This suggests that post-training cerebral reactivation is modulated by the strength of the memory traces developed during the learning episode. These data provided

the first experimental evidence for a link between behavioral performance and cerebral reactivation during REM sleep.

The manuscript gathering these results is now published : Peigneux P & Laureys S, Fuchs S, Destrebecqz A, Collette F, Delbeuck X, Phillips C, Aerts J, Del Fiore G, Degueldre C, Luxen A, Cleeremans A, Maquet P. (2003) Learned material content and acquisition level modulate cerebral reactivation during post-training REM sleep. NeuroImage, 20(1), 125-134.

Next, we tested the converse hypothesis that hippocampal activity during slow wave sleep is involved in the long term consolidation of spatial (i.e., topographical) and episodic memory in humans. Indeed, multiple cells recording in animal studies suggested that hippocampal activity during slow wave sleep (SWS) participates in the plastic changes that underlie learning and memory consolidation following a spatial experience (e.g., Nadasdy et al. 1999 J Neurosci 19:9497-50; Lee and Wilson 2002 Neuron 36:1183-94). In addition, human episodic memory, i.e. autobiographical memory for events that occur in a specific spatial and temporal context, presumably builds upon the same neuroanatomical system used for spatial learning in animals (O'Keefe et al. 1998 Philos Trans R Soc Lond B Biol Sci 353:1333-40). Previous neuroimaging studies of spatial/topographical memory repeatedly described learning-related changes in the hippocampal formation and in the parahippocampal gyrus during human navigation in virtual environments (e.g., Maguire et al 1998 Science 280:921-924; Burgess et al. 2001 *NeuroImage* 14:439-453; Shelton and Gabrieli, 2002 J *Neurosci* 22:2711-7). Therefore, learning-dependent neuronal activity changes during SWS were expected in those hippocampal and parahippocampal navigation-related locations after training to a topographical memory task in which subjects learned to find their way inside a complex 3-dimensional virtual town. Our results showed that rCBF in right parahippocampal gyrus and posterior hippocampus (pSVC < .05) both increased during route retrieval in the 3-D town and is modulated by maze learning during post-training SWS in trained subjects. Moreover, route retrieval performance improved overnight in trained subjects, and the overnight performance improvement correlated with individual rCBF variations during SWS (vs. Wakefulness) in the right hippocampus and parahippocampal gyrus (*ps*SVC < .05). Hence, we showed for the first time that the amount of hippocampal activity re-expressed during slow wave sleep predicts the improvement of performance in route retrieval on the next day. These results indicate that learning-dependent modulation in hippocampal activity during human sleep reflects the offline processing of recent episodic/spatial memory traces, which eventually leads to the plastic changes underlying the subsequent improvement in performance.

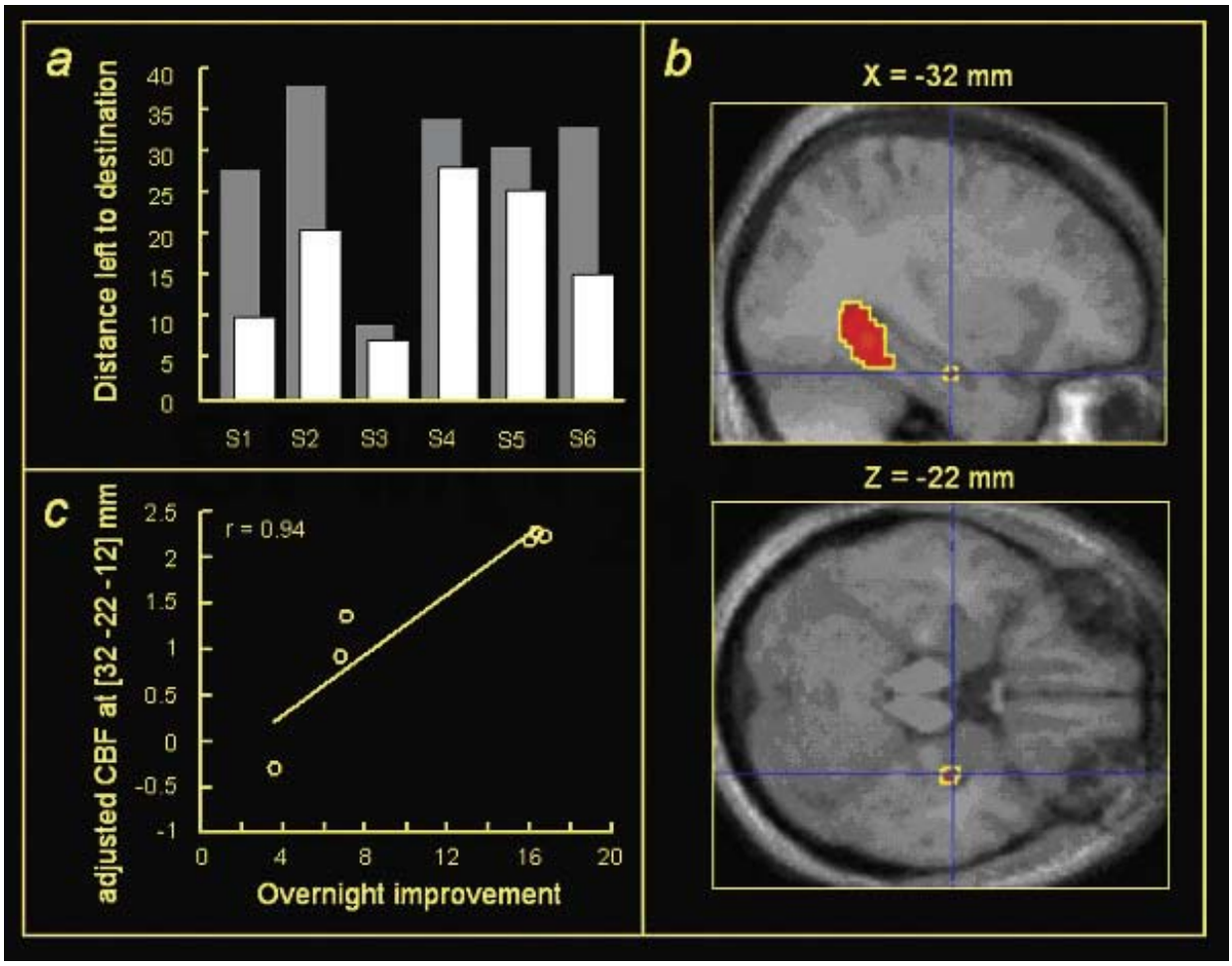

Figure 7. Hippocampal-dependent consolidation of memories during SWS. (a). Mean distance left to destination across tests of route retrieval during pre- (gray bars) and post- (white bars) sleep sessions for each trained subject (S) in group 2. All subjects improved their performance overnight. (b). Regression between overnight improvement in performance (distance left to target in pre- minus post-sleep session) and rCBF increases during SWS (versus wakefulness), superimposed on sagittal (top) and transverse (bottom) sections of the average T1-weighted MRI image of the sleeping subjects. The crosshair indicates the right hippocampus (32 -12 -22 mm; Z = 3.60). Activation in the right parahippocampal gyrus (peak at 32 -46 -10 mm; Z = 4.25) is shown on sagittal section (top). (c). Scatter plot of the correlation (r = 0.94, p < .004) between rCBF changes in the right hippocampus (at coordinates mentioned above) and overnight performance improvement (distance left to target in pre- minus post-sleep session) in route retrieval.

The manuscript gathering these results is submitted : Peigneux P and Laureys S, Fuchs S, Collette F, Perrin F, Reggers J, Degueldre C, Del Fiore G, Aerts J, Luxen A, Maquet P Spatial memories are strengthened in the human hippocampus during slow wave sleep

Taken together, these results lend support to the hypothesis that REM and non-REM sleep processes are engaged in the long-term consolidation of recent memory traces in humans. Furthermore, these data may support the dual process hypothesis which suggests that SWS is mainly involved in the processing of episodic and spatial memories whereas REM sleep facilitates consolidation of non-declarative, or procedural, memories. However, it must be kept in mind these results do not contradict the double step hypothesis which stresses the importance of the orderly succession of SWS and REM sleep in memory consolidation. To probe appropriately the second hypothesis using functional neuroimaging requires the possibility to repeatedly scan volunteers at different key points of the ultradian and circadian sleep cycle,

which was technically not possible using PET. A direct and more refined investigation of sleep using fMRI will be possible after the implementation of the EEG system in the magnet (see the Introduction section). In the meantime, we are exploring a series of new behavioural paradigms that will be implemented in sleep studies as soon as the MRI/EEG system is set up.

PUBLICATIONS

Articles

- Maquet P, Ruby P, Schwartz S, Laureys S, Albouy G, Dang-Vu T, Desseilles M, Boly M, Peigneux P (in press) Regional organisation of brain activity during paradoxical sleep. *Arch Ital Neurol*
- Maquet P, Peigneux P, Laureys S, Desseilles M, Boly M, Dang-Vu T (2003) Off-line processing of memory traces during human sleep: Contribution of functional neuroimaging. *Sleep and Biological Rhythms* 1(1), 75-83.
- Maquet P, Peigneux P, Laureys S, Boly M, Dang-Vu T, Desseilles M, Cleeremans A. (2003) Memory processing during human sleep as assessed by functional neuroimaging. *Revue Neurologique (Paris)* 159(11), S27-29.
- Maquet P, Laureys S, Perrin F, Ruby P, Melchior G, Boly M, Vu TD, Desseilles M, Peigneux P. (2003) Festina lente: evidences for fast and slow learning processes and a role for sleep in human motor skill learning. *Learning & Memory*,10(4), 237-239.
- Peigneux P & Laureys S, Fuchs S, Destrebecqz A, Collette F, Delbeuck X, Phillips C, Aerts J, Del Fiore G, Degueldre C, Luxen A, Cleeremans A, Maquet P. (2003) Learned material content and acquisition level modulate cerebral reactivation during post-training REM sleep. *NeuroImage*, 20(1), 125-134
- Maquet P, Schwartz S, Passingham R, Frith C. (2003) Sleep-related consolidation of a visuomotor skill: brain mechanisms as assessed by functional magnetic resonance imaging. J Neurosci. 23 : 1432-40

Books and Book Chapters

- Peigneux P, Laureys S, Cleeremans A, Maquet P (2003) Cerebral correlates of memory consolidation during human sleep. Contribution of functional neuroimaging. In P Maquet, C Smith, R Stickgold (Eds.) Sleep and Brain Plasticity. pp. 209-224. Oxford UK: Oxford University Press
- P Maquet, C Smith, R Stickgold (2003) Sleep and Brain Plasticity. Oxford UK: Oxford University Press

Abstracts

- Laureys S, Peigneux P, Phillips C, Fuchs S, Collette F, Degueldre C, Aerts J, Del Fiore G, Moonen G, Luxen A, Maquet P The functional neuroanatomy of human sleep as studies by PET. Federation of European Neuroscience Societies (FENS) Abstracts 1 (2002) 337
- Faymonville ME, Peigneux P, Perrin F, Boly M, Phillips C, Degueldre C, Del Fiore G, Aerts J, Luxen A, Maquet P, Lamy M, Moonen G, Laureys S Linking the EEG bispectral index (BIS) to regional brain function in normal human sleep Society for Neuroscience Abstracts 28 (2002) 16.12
- Peigneux P, Laureys S, Phillips C, Collette F, Perrin F, Fuchs S, Degeuldre C, Del Fiore G, Aerts J, Luxen A, Maquet P Spatial memories are reprocessed in the human hippocampus during slow wave sleep
  9th Annual Meeting of the Organisation for Human Brain Mapping (OHBM), June 18-22, 2003, New York City, NY, USA - NeuroImage in press
- Laureys S, Peigneux P, Destrebecqz A, Fuchs S, Collette F, Phillips C, Aerts J, Del Fiore G, Degueldre C, Cleeremans A, Luxen A, Maquet P Learning-induced changes in regional cerebral activity and connectivity during REM sleep European Journal of Neurology 10 (2003) 44, Suppl. 1

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

We continue to study conscious perception in severely brain injured patients using functional neuroimaging. And have put a special emphasis on the study of minimally conscious patients. Patients in a minimally conscious state (MCS) show very limited but definite evidence of awareness of self or environment. However, caregivers treating these patients know how difficult and frustrating it is to behaviorally evaluate or quantify their actual degree of conscious or emotional perception. Indeed, by definition, these patients cannot make any verbal or non-verbal functional communication. We have studied patients in a MCS, documented by extensive and repetitive neuropsychological evaluations, by means of FDG-PET, $H_2{}^{15}O$-PET (presentation of auditory stimuli with different emotional content and relevance) and cognitive event related potentials (ERPs). ERP studies aimed to measure P300-like responses during presentation of the patient's own name (as compared to other first names). First, we have identified the neural correlate of P300 responses to our own name in a preliminary PET study in healthy volunteers (paper submitted). Then, we have used this paradigm in brain-injured patients. In our studied MCS patients, resting cortical metabolism was less than half of normal values and comparable to values previously observed in the vegetative state. Nevertheless, auditory stimulation still induced significant cerebral activation and stimuli with emotional valence (cries and names) induced a much more widespread activation than did meaningless noise. As described in our controls, noise with spectral changes induced right-predominant superior temporal cortex activation (paper in preparation). Presentation of cries, a nonverbal universal emotional stimulus, activated widespread temporal and insular areas but not the amygdala. Cries are known to result in robust activations (regardless of attentional state) in auditory cortices (superior temporal sulcus), insula and amygdala. The amygdala are well known to be involved in emotional processing. Most importantly, presentation of the patient's own name activated the most extended neural network (precuneus, anterior cingulate/mesiofrontal, right temporo-parietal, left dorsolateral prefrontal and bilateral angular gyri) thought to be involved in reflective self-consciousness (figure 8).

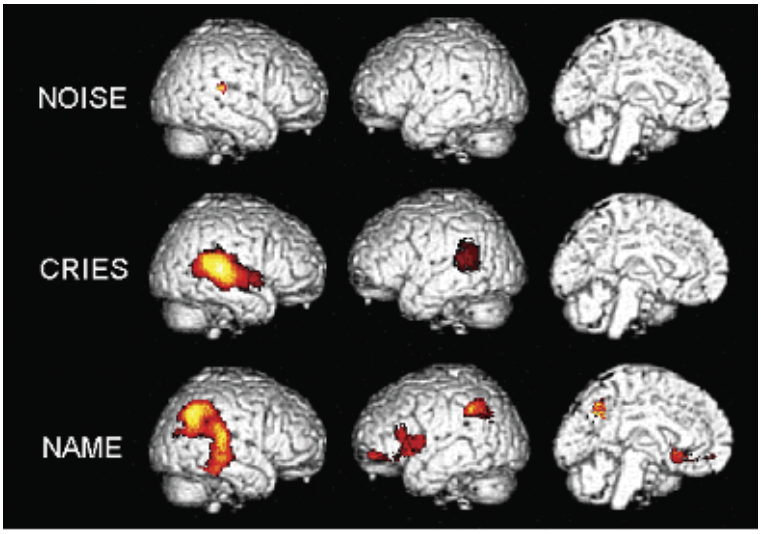

Figure 8. Brain areas that showed activation (compared to rest) during presentation of noise, infant cries and the patient's own name projected on a spatially normalized 3D-MRI template.

Independently obtained ERPs showed preserved P300-like responses to patients' own name during PET scanning (figure 9). Compared to our controls, its latency was 200 ms delayed which might be explained by a prolonged lexical access. Our own name is intrinsically meaningful because of its personal significance and repetition along life. Its powerful detection has also been shown in other situations of reduced consciousness. In superficial sleep and in end-stage dementia it is known to be the most potent stimulus to elicit discriminative responses and in patients awakening from general anesthesia, its presentation is more effective than a noise or painful stimulus. We have previously shown that in the vegetative state, auditory stimulation activates primary auditory cortices but not higher-order associative areas from whom they are disconnected. The observed context-dependent higher-order auditory processing in MCS patients shows that content does matter when talking to these patients and encourage the use of neuro-stimulation programs in this very challenging patient population (paper submitted for publication).

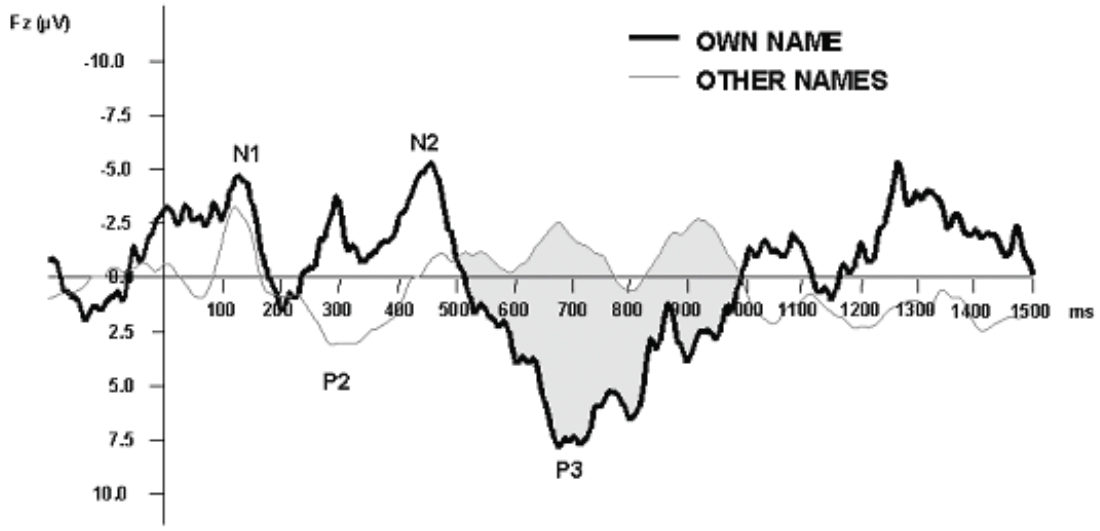

Figure 9. Event related potentials in response to the patient's own name (thick line) and to other names (thin line). Data are averages from 3 sessions (each containing 2x27 stimuli) obtained during PET scanning.

The goal for 2004 is to study residual brain function and plasticity in brain-injured patients by use of functional MRI. We are now preparing the use of these more powerful methods to confirm whether the observed lack of amygdalar processing is characteristic of the MCS or related to the limited sensitivity of our used H215O-PET technique.

PRIZES and AWARDS

- 2003 : Human Brain Project Travel Award, Organization for Human Brain Mapping's 9th Annual Meeting, New York, USA
- 2003 : European Federation of Neurological Societies Award, 7th Annual Meeting, Helsinki, Finland

PUBLICATIONS

Articles:

- Brain, conscious experience and the observing self
  Baars B, Ramsoy T, Laureys S
  Trends in Neurosciences, 26 (2003) 671-675 [impact factor 14.474]
- 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, in press [impact factor 4.393]
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  Robe PA, Dubuisson A, Bartsch S, Damas P, Laureys S
  Journal of Neurology Neurosurgery and Psychiatry 74 (2003) 1157-1158 [impact factor 3.041]
- Brain function during catatonic stupor and after clinical recovery
  De Tiège X, Laureys S, Massat I, Bier JC, Lotstra F, Berré J, Mendlewicz J, Goldman S
  Journal of Neurology Neurosurgery and Psychiatry 74 (2003) 1003-1004 [impact factor 3.041]
- Functional neuroimaging of auditory processing
  Laureys S, Salmon E, Goldman S, Majerus S
  Acta Otorhinolaryngologica Belg 57 (2003) 267-273

Other publications:

- Book review:
  The Vegetative State: Medical Facts, Ethical and Legal Dilemmas by B Jennett
  Laureys S
  Advances in Clinical Neuroscience and Rehabilitation 3 (2004) 14
- Coma
  Laureys S, Faymonville M-E, Maquet P
  Encyclopaedia Universalis, in press, Paris, France
- Renouer avec les consciences emmurées
  Laureys S
  Nouvelles Clés 37 (2003) 46-50

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- 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
  Edited by Machado C, Shewmon DA, Kluwer Academic/Plenum Publishers, New York, in press
- Hysteria and catatonia
  De Tiège X, Goldman S, Laureys S
  In: Nuclear Medicine in Psychiatry
  Edited by Audenaert K, Otte A, Dierckx RA, van Heeringen K, Springer-Verlag, Berlin, in press

Abstracts & Conference Proceedings

- EEG bispectral index correlates with regional changes in brain function
  Laureys S, Peigneux P, Faymonville ME, Perrin F, Degueldre C, Del Fiore G, Luxen A, Moonen G, Lamy M, Maquet P
  9th Annual Meeting of the Organisation for Human Brain Mapping (OHBM), June 18-22, 2003, New York City, NY, USA - NeuroImage CD
- Auditory processing in severely brain-injured patients: differences between the minimally conscious and the vegetative state
  Boly S, Faymonville ME, Damas P, Lambermont B, Del Fiore G, Degeuldre C, Aerts J, Luxen A, Moonen G, Maquet P, Laureys S
  9th Annual Meeting of the Organisation for Human Brain Mapping (OHBM), June 18-22, 2003, New York City, NY, USA - NeuroImage CD
- Neural mechanisms involved in the detection of our first name: a combined ERPs and PET study
  Perrin F, Maquet P, Peigneux P, Degeuldre C, Phillips C, Aerts J, Del Fiore G, Luxen A, Laureys S
  9th Annual Meeting of the Organisation for Human Brain Mapping (OHBM), June 18-22, 2003, New York City, NY, USA - NeuroImage CD
- Differences in brain metabolism between patients in coma, vegetative state, minimally conscious state and locked in syndrome
  Laureys S, Faymonville ME, Ferring M, Schnakers C, Elincx S, Ligot N, Majerus S, Antoine S, Mavroudakis N, Berré J, Luxen A, J-L Vincent, Moonen G, Lamy M, Maquet P
  7th Congress of the European Federation of Neurological Societies, August 30-September 2, 2003, Helsinki, Finland
- Neural mechanisms involved in the detection of our given name: a multi-modal study using event related potentials and positron emission tomography
  Perrin F, Peigneux P, Maquet P, Degueldre C, Luxen A, Phillips C, Aerts J, Del Fiore G, Laureys S
  7th Congress of the European Federation of Neurological Societies, August 30-September 2, 2003, Helsinki, Finland
- Learning-induced changes in regional cerebral activity and connectivity during rem sleep
  Laureys S, Peigneux P, Destrebecqz A, Fuchs S, Collette F, Phillips C, Aerts J, Del Fiore G, Degueldre C, Cleeremans A, Luxen A, Maquet P
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- Neuropsychological testing in the locked in syndrome : preliminary results from a feasibility study
  Schnakers C, Majerus S, Van Eeckhout P, Peigneux P, Goldman S, Laureys S
  56th Annual Meeting of Belgian Psychological Society (BPS) International Symposium, May 23, 2003, Vrije Universiteit Brussel

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

Language

Modulation of brain activity during phonological learning: a PET familiarization paradigm.

While many studies have explored the neural substrates of language processing, the cerebral correlates of language learning have received very little attention. The aim of this study was to investigate the neural processes of a very basic language learning process: phonological familiarization. We measured brain activity in 12 French-speaking young adults for the repetition of auditorily presented words and nonwords before and after repeated exposure to their phonological form. The nonword phoneme combinations were either frequent (HN) or infrequent (LN) relative to the phonological structure of French. After familiarization, we observed decreased activation in the left posterior superior temporal lobe as well as in the bilateral temporal pole and middle temporal gyri, for both word and nonword stimuli (see figure). Interaction analysis however showed that, for LN nonwords, this decrease of activation was significantly less important in the bilateral posterior superior temporal lobe, relative to both the word and HN nonword conditions (Figure 10). Furthermore after familiarization, activity in the bilateral posterior lobe was most reduced in those participants that showed the strongest behavioral familiarization effects (Figure 11). The results suggest that phonological familiarization is related to decreased activity in sublexical and lexical phonological processing areas, and that the extent of this decrease in brain activity interacts with initial stimulus familiarity.

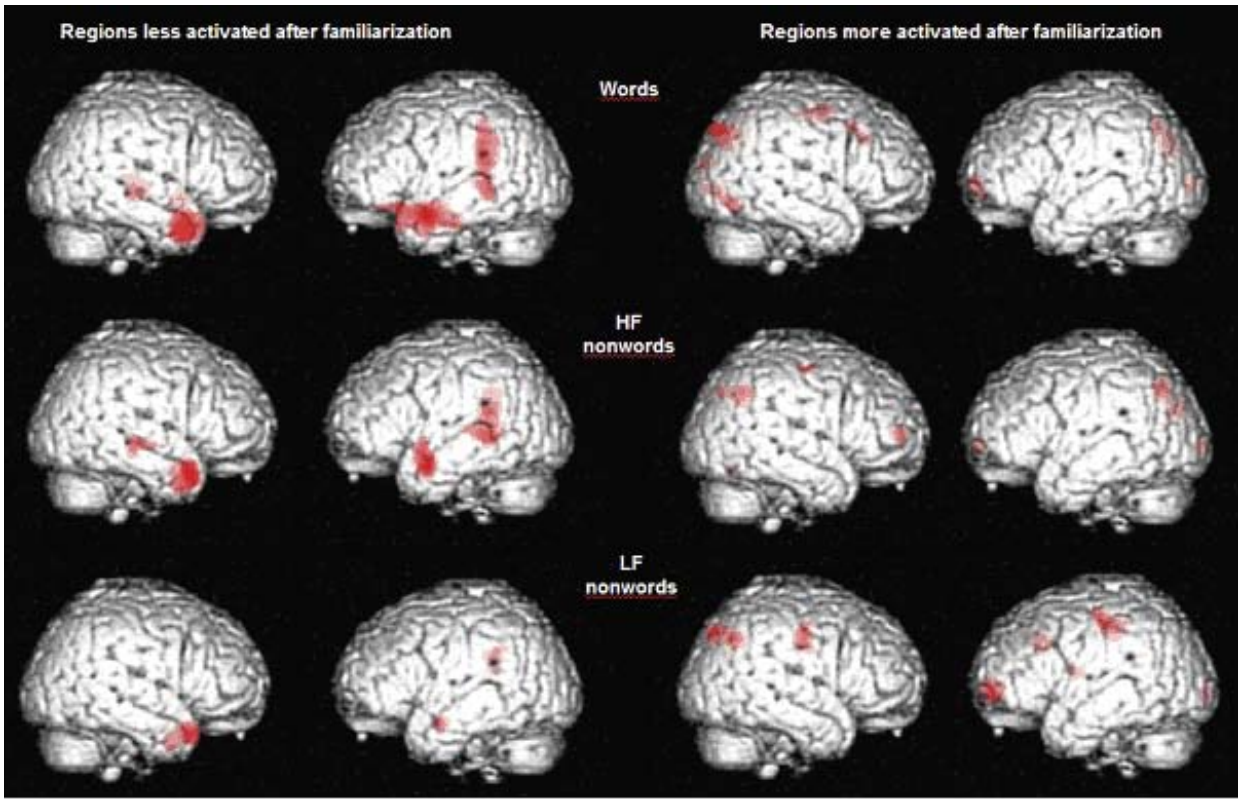
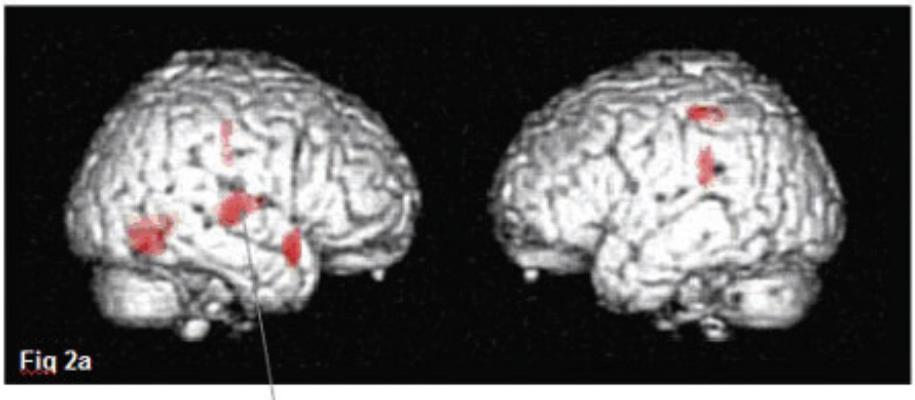


Figure 10. Regions less or more activated after familiarization, as a function of stimulus condition (p<.0001 uncorrected, voxel threshold: k=50).



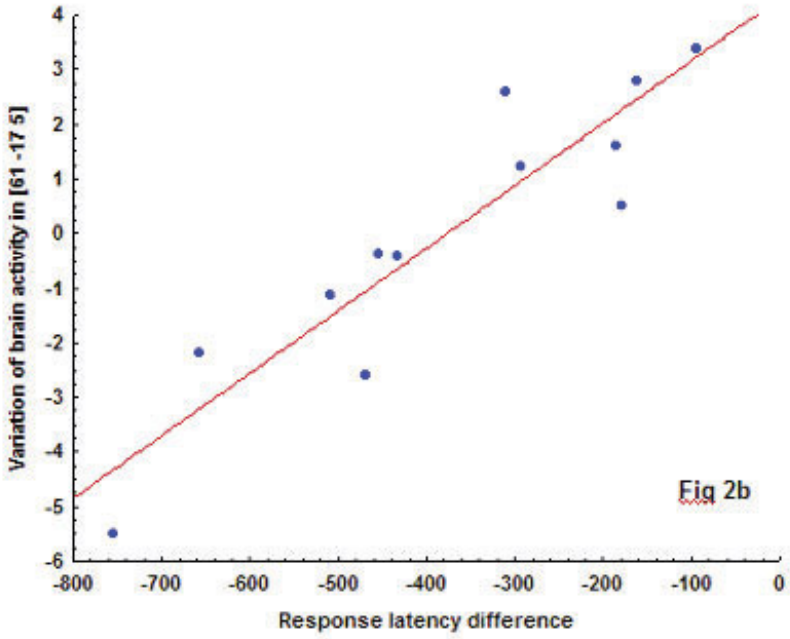


Figure 11. Regions that correlate with the reduction of response latencies for LN nonwords after familiarization ($p < .0001$, uncorrected; spatial extent threshold: $p<.05$)

The paper is submitted for publication

Executive functioning

The simultaneous processing of multiple tasks

The ability to coordinate concurrent cognitive processes is a crucial executive function. This function has commonly been investigated by using dual-task paradigm in which two tasks are performed successively and thereafter simultaneously. At present, the neural substrates of dual-task coordination are not well established: some studies suggested that the coordination function involves increased activity in specific areas of the prefrontal cortex (1) while other studies considered that the coordination function relates to the interplay of various areas already involved during the execution of the single tasks (2). These studies, however, used complex cognitive tasks which could have induced a prefrontal activation at the single task level. Consequently, the aim of the present study is to explore cerebral areas involved in dual-tasks coordination using tasks which do not involve any prefrontal activation at the single task level. We were also interested to compare cerebral areas involved in dual task coordination to those involved in the integration of simultaneously presented information.

A $H_2{}^{15}O$-PET study was performed in 13 right-handed volunteers. The paradigm included two single (visual and auditory) detection tasks, a dual-task (simultaneous visual AND auditory detection), and baseline tasks. In the visual task, subjects were presented crosses and had to decide whether the cross appeared in the inferior or superior part of the screen by pressing a key. In the auditory task, subjects had to decide if a tone was high- or low-pitched by pressing a key. In the dual-task condition, a cross and a tone were presented simultaneously, the subjects having to detect both the position of the cross and the pitch of the tone (also by pressing a key). In the integration task, subjects had to integrate auditory and visual information to decide whether the two stimuli were congruent or not. A congruent pair was defined as composed of a cross presented in the upper portion of the screen and a high-pitch tone (both items are "at the top") or a cross presented in the lower part of the screen paired with a low-pitch tone (both items are "at the bottom"). Baseline tasks consisted of neutral visual (crosses in the middle of the screen) or auditory (middle-pitched tones) stimuli after which subjects again had to press a key. Data were analyzed using SPM99.

The comparison of each single task to the baseline tasks showed no activity in prefrontal areas ($p<0.0001$, uncorrected for multiple comparisons) while the comparison of the single tasks to the dual task condition demonstrated foci of activity in the left inferior parietal gyrus (BA 40; peak voxel: x=40, y=-58, z=38; $p<0.05$, corrected for multiple comparison), and the left middle frontal gyrus (BA 9/46; peak voxel: x=-40, y=18, z=24; BA 10/47; peak voxel: x=-46, y=50, z=-14; $p<0.05$; BA 6; peak voxel: x=-28, y=6, z=46 corrected for multiple comparison) (figure 12). Globally similar regions were found for the integration task, except that the inferior parietal gyrus was not recruited.

These results confirm the implication of prefrontal cortex during dual-task coordination. Indeed, the observed prefrontal activity cannot be attributed to the realization of the single tasks, since no prefrontal activity was found when single tasks were compared to baseline. Moreover, the involvement of a parietal area in the dual-task condition is in agreement with the hypothesis of a parieto-frontal network sustaining executive functioning. More precisely,

BA 9/46 was associated with the processing of interfering information, BA 10/47 with selection processes, BA 6 with articulatory rehearsal and BA 40 with attentional shifting during our simple dual task. As indicated previously, similar regions were found for the integration task, except for the inferior parietal gyrus. These results confirm the hypothesis according to which the left prefrontal cortex is implicated in dual-task performance.

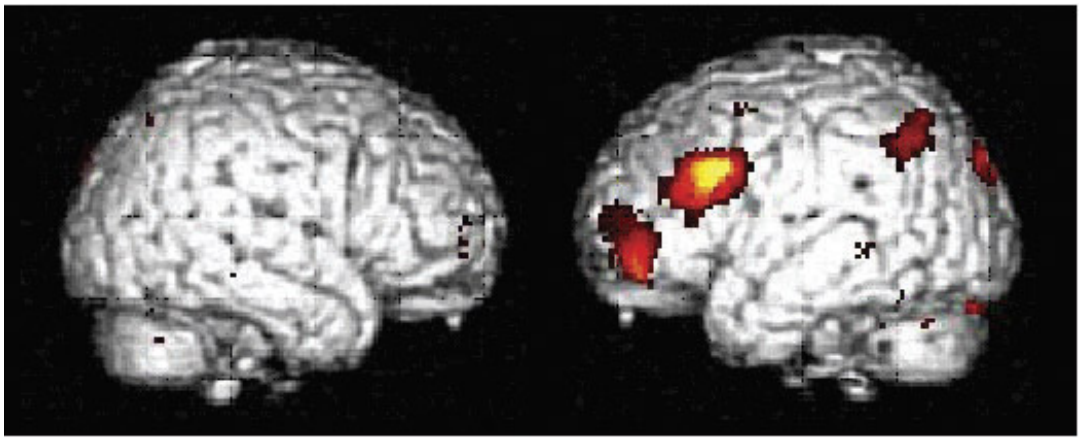


Figure 12.Brain activation when dual task processing is compared to discrimination tasks

The paper is submitted for publication

Sustained and transient cerebral responses during an updating task.

Previous neuroimaging studies have explored the executive process of updating using running span tasks (requiring subjects to watch strings of items of unknown length and to recall serially a specific number of the lastly presented items). These studies showed patterns of cerebral activity both in prefrontal and parietal areas. However, these results were obtained with positron emission tomography and the temporal dynamics of cerebral areas involved in the running span task could not be assessed. Consequently, we re-explored the cerebral areas associated to this task with functional magnetic resonance imaging (fMRI). We used the temporal resolution of this technique to distinguish cerebral areas continuously activated during trials requiring updating from those transiently activated during the presentation of items to update.

Nineteen right-handed healthy volunteers (nine females) took part in the experiment (age range: 20-26 years). Two kinds of trials were presented: *storage and updating*. For all trials, strings of consonants were visually presented that subjects were instructed to rehearse silently and to remember serially. At the end of each trial, a consonant was displayed with a digit and subjects had to indicate if the letter was at the position specified by the digit. In the *storage* trials, sequences of four consonants were displayed that subjects had to memorize. In the *updating* trials, lists of 6, 8 or 10 consonants were presented. Subjects were not informed of the length of the trial and they were asked to remember only the last four items. *Storage and updating* trials were randomly presented during the scanning session. Data were acquired on a 3T scanner using a T2* sensitive gradient echo spiral sequence (TR=2130msec, TE=, 40msec, 32 contiguous 3-mm thick transverse slices). In each session 999 functional volumes were

obtained. Data were analyzed using SPM2 and a random effects model at the group level. A mixed model was used: for each trial, blocks correspond to the period during which subjects have to update information (namely, from the five consonant to the end of the trial) while events correspond to the specific moment where subjects have to update information.

The average accuracy for the different trials was the following: length 4=87%, length 6=87%, length 8=84%, length 10=85%. Several regions were continuously activated during the updating process: the pre and post central areas ($p<0.05$, corrected for multiple comparisons); the left frontopolar, the right middle and bilateral inferior frontal gyri, the right parietal cortex and the bilateral cerebellum ($p<0.05$, uncorrected for multiple comparisons). Regions with transient activity associated to updating were the insula and entorhinal cortex (right>left) and the posterior cingulate ($p<0.05$, corrected for multiple comparisons); the precental gyrus and the cerebellum bilaterally, the left angular gyrus and the anterior cingulate ($p<0.05$, uncorrected for multiple comparisons).

These results indicate that two distinct cerebral networks can be associated to the running span task. Indeed, some regions demonstrate a sustained activity when subjects have to update information (figure 14) while others are only transiently activated when the items to update are presented (figure 13). Regions with sustained activation were previously found in tasks requiring both storage and manipulation of information in working memory while those with transient activity were previously associated to encoding and sequential processing of information.

Figure 13. Cerebral areas transiently activated by the updating process

Figure 14. Cerebral areas continuously activated by the updating process

Physiopathology of dementia

In a multicenter study, FDG-PET images in a population of twenty-nine patients with frontotemporal dementia (FTD) were respectively compared to controls with similar age from each center. A conjunction analysis led to identification of the ventromedial frontopolar cortex as the single region affected in each and every FTD patients. This precise regional metabolic impairment should be integrated with recent neuropsychological researches, such as those showing that the ventromedial frontal cortex is critically involved in decision making processes based on personal experience, feelings of rightness or social knowledge, processes that are characteristically impaired in FTD (figure 15).

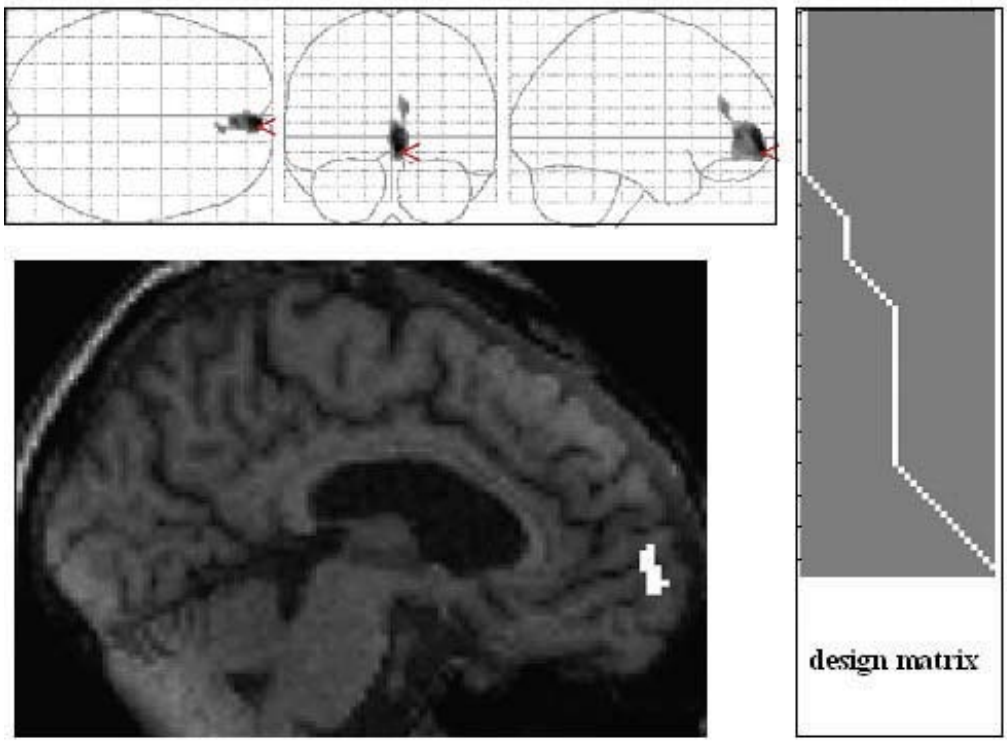


Figure 15. Ventromedial prefrontal cortex metabolically impaired in all FTD patients

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Progress Report of the Research Group of

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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/CRD-NRG. To examine the biological effect of Nrg1, we added soluble ErbB3 (sErbB3) to growing neurospheres. This inhibitor decreased NP mitosis and increased their apoptosis, resulting in a significant reduction in neurosphere size and number. When NP were induced to migrate and differentiate by adhesion of neurospheres to the substratum, the level of type III NRG-1 isoforms detected by RT/PCR and Western blot decreased in the outgrowth in parallel with a decrease in Nrg1 fluorescence intensity in differentiating astrocytes, neurons and oligodendrocytes. Pretreatment of growing neurospheres with sErbB3 induced a three fold increase in the proportion of oligodendrocytes generated from migrating NP after neurosphere adhesion. This effect was not observed with an unrelated soluble receptor. Addition of sErbB3 after adhesion did not change the proportion of oligodendrocytes in the neurosphere outgrowth but enhanced their expression of galactocerebroside and myelin basic protein. We propose that both Type III Nrg1 signaling and released soluble ErbB receptor may modulate oligodendrocyte development from NP (Calaora et al., 2001).

We have expressed as a recombinant protein the intracellular domain of SMDF/CRD-NRG and we have purified it. Then we raised a rabbit polyclonal antiserum against this intracellular part of SMDF/CRD-NRG (SMDF-IC). Using purified IgG, we observed in immunocytofluorescent studies that SMDF-IC is present at plasmic membrane level in proliferating NP and in nuclei of differentiating neurons and oligodendrocytes but not in astroglial nuclei. Indeed, SMDF-IC exhibits a strong nucleus localization signal. We made the hypothesis that there is a cleavage of SMDF/CRD-NRG and the SMDF-IC is then transferred to nucleus. This nuclear translocation is followed by a oligodendroglial or a neuronal differentiation. It has been recently demonstrated that SMDF/CRD-NRG is cleaved at the plasmic membrane level (Frenzel and Falls, 2001). Moreover, the SMDF-IC could be observed in the nucleus of neurons (Bao et al., 2003). So it is important to look for the protein(s) interacting with SMDF-IC and which could be translocated into the nucleus with SMDF-IC and there behave(s) like a transcription factor. We have started the co-immunoprecipitation studies using our polyclonal anti-SMDF-IC.

More recently, we have demonstrated that NRG-2 but not NRG-3 is expressed in proliferating neurospheres. No NRG-2 expression is observed in NP after 5 days of differentiation. The expressed NRG-2 isoforms is related to two already described isoforms (Busfield et al., 1997; Higashiyama et al., 1997). Inintact embryonic striata, a transmembrane isoform is expressed. Proliferating NP expressed in culture both an transmembrane and a secreted form of NRG-2. The expression f 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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Identification and characterization of an adult hippocampal multipotent progenitor cell in the subventricular zone

Belachew, S. and Nguyen, L.

Two major multipotent neural stem cell (NSC) phenotypes have been described in the subventricular zone (SVZ), based on expression of the intermediate filament protein glial fibrillary acidic protein (GFAP; Doetsch et al., 1999 and 2002; Capela and Temple, 2002). Type B cells express GFAP and give rise to the rapidly dividing, transit-amplifying Type C cells, which are not immunopositive for GFAP (Doetsch et al., 1999). It has been demonstrated that SVZ Type C cells: i) are able to generate neurospheres in the presence of epidermal growth factor (EGF); ii) express the Dlx2 transcription factor and the EGF receptor (EGF-R), and iii) are the direct precursors of neurons generated in the olfactory bulb (Doetsch et al., 1999; 2002).

Adult NSCs express the Lewis X (LeX) carbohydrate antigen, which is detected in both $GFAP^+$ and GFAP-negative cells of the SVZ (Capela and Temple, 2002), indicating that this brain region contains a LeX^+/GFAP-negative cell population with neurogenic potential. Since Type C cells are a GFAP-negative and highly proliferative cell population, it is likely that the LeX^+/GFAP-negative stem cells are in fact Type C progenitors (Capela and Temple, 2002; Doetsch et al., 2002).

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 aimed at determining which effect Reelin, a protein of the extracellular matrix expressed in the developing cortex, has on the morphology and the biochemical maturation of the radial glia. Reelin is also known for its implication in the control of neuronal migration and the formation of cortical layers. We have shown that Reelin controls the production of the radial fibre of the radial glial cells and their expression of BLBP, a protein that plays an important role in the control of neuronal migration. This effect is observed in the cortex but not in the basal ganglia. It is a direct effect of Reelin on the radial glia which implies a signaling mechanism that uses the adaptator protein Dab1. This work has been published 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, J.M., Hans, G. and Moonen, G.

Our laboratory has collaborated for several years with a team at UCB for the characterization of the mode of action of a new antiepileptic drug, levetiracetam (Keppra). Within this framework we carried out many measurements of the parameters which characterize the interaction of a radioactive analogue of levetiracetam, tritiated UCB 30889, with its binding sites on cultures of nervous cells. The dissociation constants (Kd) and numbers of binding sites (Bmax) were thus determined on cultures of cortical neurons, cerebellar granule cells and cortical astrocytes of mouse taken during the perinatal period. Furthermore, we took part in an effort to identify the protein which constitutes the binding site in the cortex of adult rat. For this purpose, we have made solubilized proteins preparations after cross linking of 3H-UCB 30889 with rat brain synaptosomes. These protein extracts were then analyzed by electrophoresis with counting of the radioactivity in the individual protein bands. A protein of about 100 kDa was thus identified but its nature has not been determined yet. An article presenting 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, Lefebvre P.P., Lallemend F., Belachew,S., Rigo, J.M., Nguyen, L. and Moonen, G.

Most hearing loss results from lesions of the sensory cells and/or neurons of the auditory portion of the inner ear. Characterization of the genes that regulate the development of the inner ear and its response to trauma has been helpful in designing strategies for enhancing protection of the inner ear and for inducing hair cell regeneration. In the embryonic day 19 organs of Corti, we showed that roscovitine, a chemical inhibitor of cyclin-dependent kinases, significantly increased the number of hair cells (HCs) and corresponding supporting cells (SCs) by triggering differentiation of precursor cells without interacting with cell proliferation. The effect of roscovitine was mimicked by other CDK1, 2, 5, 7, inhibitors, but not by CDK4/6 and MAP kinase pathway antagonists. Immunohistochemical analysis indicated that roscovitine specific intracellular targets, CDK1, 2, 5, 7 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 CDKs activity was higher and more roscovitine-sensitive at developmental stages that are selectively permissive for the emergence of supernumerary cells. These results suggest that cyclin-dependent kinases 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 corresponding SCs, presumably from the Hensen's cells progenitors. The demonstration of roscovitine-induced HC differentiation might open prospects for using this apparently well tolerated drug to devise new strategies to stimulate HC regeneration in the mammalian organ of Corti.

In parallel, we try to develop new in vitro approaches to prevent spiral ganglion neuronal cell death. We have investigated the ability of substance P (SP) to protect 3-day-old (P3) rat spiral ganglion neurons (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 NK1 antagonist, WIN51708, blocked the survival promoting effect of SP. 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 antagonized by specific inhibitors of second messengers, including 1.2-bis-(O-aminophenoxy)-ethane-N,N,N',N'-tetra-acetic acid (BAPTA-AM) to chelate cytosolic Ca2+, the protein kinase C (PKC) inhibitors bisindolylmaleimide

I, Gö6976 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 4ß-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 TFD induced SGN cell death via pathways involving PKC activation, Ca2+ signaling and MAPK/ERK activation, which can be accounted for by an inhibition of caspase activation.

Neurotransmitters as early signals for central nervous system development
Nguyen, L., Belachew, S., Malgrange, B., Rogister, B., Moonen, G. and Rigo, J.M.

During brain ontogenesis, the temporal and spatial generation of different types of neuronal and glial cells from precursors occurs as a sequence of successive progenitor stages whose proliferation, survival and cell fate choice are controlled by environmental and cellular regulatory molecules. Neurotransmitters belong to the chemical microenvironment of neural cells, even at the earliest stages of brain development. It is now well established that specific neurotransmitter receptors are present on progenitor cells of the developing nervous system and could play, during development, a role that has remained unsuspected until recently.

Gamma-aminobutyric acid (GABA) and its type A receptor ($GABA_AR$) are present in the immature central nervous system (CNS) and may function as growth-regulatory signals during the development of embryonic neural precursor cells. In the present study, based on their isopycnic properties in a buoyant density gradient, we developed an isolation procedure that allowed us to purify proliferative neural precursor cells from early postnatal rat striatum, which expressed the polysialylated form of the neural cell adhesion molecule (PSA-NCAM). These postnatal striatal PSA-NCAM+ cells were shown to proliferate in the presence of epidermal growth factor (EGF) and formed spheres that generated preferentially neurons in vitro. We demonstrated that PSA-NCAM+ neuronal precursors from postnatal striatum expressed $GABA_AR$ subunits in vitro and in situ. GABA elicited chloride currents in PSA-NCAM+ cells by activation of functional $GABA_AR$ that displayed a typical pharmacological profile. $GABA_AR$ activation in PSA-NCAM+ cells triggered a complex intracellular signaling combining a tonic inhibition of the mitogen-activated protein kinase cascade and an increase of intracellular calcium concentration by opening of voltage-gated calcium channels. We observed that the activation of $GABA_AR$ in PSA-NCAM+ neuronal precursors from postnatal striatum inhibited cell cycle progression both in neurospheres and in organotypic slices. Furthermore, postnatal PSA-NCAM+ striatal cells synthesized and released GABA, thus creating an autocrine/paracrine mechanism that controls their proliferation. We showed that EGF modulated this autocrine/paracrine loop by decreasing GABA production in PSA-NCAM+ cells. This demonstration of GABA synthesis and GABAAR function in striatal PSA-NCAM+ cells may shed a new light in the understanding of key extrinsic cues that regulate the developmental potential of postnatal neuronal precursors in the CNS.

We next reported by immunocytochemical analysis that ionotropic glycine receptors are expressed in neurogenic progenitors purified from the newborn rat striatum and expressing the polysialylated form of the neural cell adhesion molecule, 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 these cells. 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. The roles of those glycine receptor remain to be elucided, and our futures studies will be dedicated to untangle their function (s) in the regulation of proliferation, and/or differentiation of neurogenic progenitors within the developing striatum.

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

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

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

We have established the culture method in order to isolate and cultivate mesenchymal stem cells from adult rat and mice. We have characterized those cells in an immunological but also on a functional point of view and we demonstrate that those mesenchymal stem cells could differentiate into adipocytes, osteocytes and chondrocytes. In particular culture conditions they become able to express the nestin, an intermediate filament protein which is highly expressed in immature cells of the nervous system. We demonstrated that lysophosphatidic

acid and thrombin could repress this expression. Moreover the nestin expression is only observed in mesenchymal stem cell cultures which are old enough in culture: before ten passages, no nestin could be seen in favourable culture conditions. It means that mesenchymal stem cells need to mature or to transform in vitro, in order to acquire the ability to express nestin. This nestin expression property is a hallmark of other modifications: if mesenchymal stem cells are able to expressed nestin, they can form clusters in non-adherent culture conditions, just like neural stem cells do.

When nestin(+) but not nestin(-) mesenchymal stem cells are co-cultivated with neural stem cells in suspension, they rapidly form heterogenous spheres. If those spheres are plated on adherent surface for 5 days, one can observe a cell differentiation and 40 % of original mesenchymal stem cells express two astroglia-specific markers : GFAP and GLAST. We demonstrated using two procedures that those GFAP(+) cells are from stromal origin and are not a consequence of a cell fusion between a mesenchymal stem cell and a neural stem cell. Finally, nestin(-) mesenchymal stem cells will not express GFAP when they are co-cultivated with neural stem cells and a direct cell-to-cell contact is necessary to observe a GFAP expression by nestin(+) mesenchymal stem cells (MSCs)(Wislet-Gendebien et al., 2003).

More recently, we started to co-cultivate nestin(+)MSCs with granule cerebellar neurons and in those condtions, 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, …. . I was recently reported that in vivo MSCs are able to fuse with neurons (Weinmann et al., 2003; Alavarez-Dolado et l., 2003). 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. 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.

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Selectivity of monkey V4 neurons for kinetic patterns

Background

The single-cell study of selectivity of V4 neurons for kinetic patterns serves as test bed for the comparison single cells and fMRI recorded in the same species under the same conditions with the same stimuli. We have datasets of fMRI data obtained with kinetic gratings and kinetic stimuli (Fize and coworkers), we need to build up a comparable dataset of single-cell responses in order to begin to understand the link between selectivity as assessed in single-cell studies and fMRI signals which reflect primarily levels of activity of large populations of neurons. This comparison is the more important that our earlier efforts to compare fMRI in the awake monkey with deoxyglucose - the purpose of a complete EU project (Mapawamo), have been stopped in their tracks due to the lack of tritium sensitive film. The companies producing such film have withdrawn the film we used and all substitutes so far are worthless. Thus we have abandoned this comparison effort, making the comparison with single-cells results even more crucial.

On the other hand we have begun the analyze the vast amount of fMRI data on kinetic responses, an effort slowed down by the move of D. Fize back to Toulouse, where he has now a permanent research position and very much involved in setting up the 3T magnet for monkey fMRI. What we have found is that there are systematic differences between the local maximum approach, typical of SPM and that we traditionally use in our monkey fMRI analysis, and the ROI approach. When defining a relatively small ROI (40 or so voxels) we fail to observe the significantly larger activation by kinetic gratings compared to transparent motion, we do observe in the local maximum in V4 obtained with that contrast. Thus it seems that the sensitivity for kinetic gratings may be patchy. After all our former 2DG studies (Vanduffel et al., 2002; Tootell et al., 2004) have shown several examples of such patches of activity in V4 and IT cortex. This means that we have to increase our sampling efforts in the single-cell studies so as to ensure we record from enough of these patches.

Results

Given the need to sample widely from V4 (because of the patchiness of selectivity) we have continued to record from our first monkey and started to record from a second monkey. Our careful sampling strategy was detailed in the previous report. The sample now stands at 407 neurons tested in the two animals. Of these 163 (40%) were selective for orientation of a luminance grating (assessed by ANOVA) and 45 (11%) were selective for kinetic gratings, meeting all criteria of invariant tuning for the two types of kinetic gratings (parallel and orthogonal). Furthermore 269 of these neurons were tested with shapes (the same as used in fMRI and IT single cell study of Sáry et al., 1993). 132 of them (49%) were selective for luminance defined shapes and 67 (25%) for kinetic shapes.

In addition to studying the incidence of neurons selective for kinetic patterns, we also compiled the overall responses to the different stimuli compared in the single-cell recording and the fMRI. The average response of the 407 V4 neurons to luminance gratings equalled 16.3 spikes/s, to kinetic gratings 10 spikes/s, to transparent motion 8.1 spikes/s and to uniform motion 9.7 spikes/s. For the 269, neurones tested with shapes these values were 19.3 spikes/s for luminance defined shapes, 12.4 spikes/s for kinetic shapes, 3.6 spikes/s for the transparent control and 6.1spikes/s for the luminance control.

The preliminary results from the first monkey were presented at the Neuroscience meeting in November 2003 in New Orleans (Mysore et al., 2003 Soc.Neurosci.Abstr. 439.5).

Perspectives

The future plans call for sampling further in the two monkeys, probably until we reach 500 or so tested cells. Most analysis is done in parallel with the recording so the analysis and writing of the paper should go rather smoothly. This will be done in parallel with the writing of the fMRI paper on the same topic.

Given the importance of patchiness and as intermediate recording between the single cells and fMRI we have started to adapt the experimental set-up so that we can record single units as well as local field potentials with micro-electrodes. LFP may be a more efficient way to document the patchiness directly rather than the single-cell recordings.

Finally this work is integrated in our efforts to understand the changes during evolution between monkeys and humans in the region corresponding to V4. Other experiments performed in the lab bear on the same issue. For example in humans we recently obtained crucial evidence that much of the human LOS region (located in the same relative position as V4d in monkeys) responds only to small central stimuli. Perhaps this is the way forward in the sense that much of LOS is really the homologue of the central representation of V4.

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  (33rd Society for Neuroscience Meeting, New Orleans, November 8-12, 2003)

Progress Report of the Research Group of

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Pathogenesis of cellular dysfunction and death in frataxin deficiency

Activities during the second year of FMRE support have been focused on the analysis of the primary function of frataxin and on the development of cellular models. A project on celleular therapy of Friedreich ataxia (FA) has been started.

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

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

Specific aims 1 and 2

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

After the important discoveries on the primary function of frataxin that were made in 2002 by yeast genetics laboratories (in particular by R. Lill in Germany), that indicate a direct role in the intramitochondrial synthesis of of iron-sulfur clusters (ISC), we wished to confirm the yeast findings in mammalian cells. In particular, we wished to confirm a physical interaction, between frataxin and other proteins involved in the early phase of ISC synthesis, namely IsuI and NsfI.

We performed immunoprecipitation and pull-down assays using mouse mitochondrial extracts, and our preliminary data are in agreement with such a physical interaction, at least with the scaffold protein where ISC are assembled, IsuI.

Second, we wished to investigate whether the expression of frataxin and other ISC synthesis proteins is co-regulated. Our preliminary data show that frataxin, NsfI and IsuI are expressed at different levels in different mouse and human cell types, but they maintain their relative abundance, suggesting that their expression is co-regulated.

Third, we are currently analyzing levels of IsuI and NsfI proteins in mice that overepress frataxin (two different transgenic lines obtained in my former Montreal lab) as well as in mice that have a partial deficiency of frataxin.

Fourth, we have started using a siRNA approach to induce deficiency of frataxin and other ISC synthesis proteins in cultured cells. Preliminary experiments with direct RNA transfection have been very promising, so constructs to generate stably transfected cell lines (neuroblastoma) with inducible expression of these siRNAs are being generated.

Fifth, we have started a collaboration with the group of Dr. Andreu in Barcelona to study whether frataxin affects iron-dependent changes in mitochondrial DNA conformation and transcription previously described by this group. This study is particularly interesting because, if any effect is detected, it would on the one hand support a direct frataxin-iron interaction and on the other hand indicate a so far unexplored pathogenic mechanism in frataxin deficiency.

Specific aim 3

The collaboration with Professor Cocozza of the University of Naples for a study on the activation of the stress kinases pathways (MKK4, JNK, ERK, p38) in frataxin-deficient cells, mentioned in the previous report, is ongoing. However, we decided to focus our efforts of the neuroblastoma cells with inducible anti-frataxin siRNA that are currently being prepared rather than the more artificial P19 system that we previously envisaged.

Specific aim 4

The cDNA microarray gene expression study in frataxin deficient mice (knock-in / knock-out mice with 20 % of wild type frataxin levels) that not show symptoms of visible pathology, so as to avoid detecting changes due to cell loss or inflammation, is now almost completed.

As indicated in the previous report, several analyses were performed including comparison of gene expression in males and females and identification of genes that were altered in common in cervical spinal cord and brainstem. Genes with significant alterations include those within functional categories of iron homeostasis regulation and oxidative stress response, signal transduction, metabolic, and the neuronal development pathway. Results have already been communicated at international meetings and a manuscript is in preparation.

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Functional characterization of orphan G protein-coupled receptors expressed in brain.

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. Cloning and expression of orphan receptors

With the aim of identifying the natural ligands of orphan receptors, we have established over the past years a collection of cell lines expressing genes encoding putative neuropeptide receptors collected in the databases and the human genome sequencing program. Altogether, about ninety human orphan receptors have been cloned. The coding region is inserted in a bicistronic expression vector that allows to obtain high expression levels and long term stability of the cell lines. The recombinant plasmids were expressed in CHO-K1 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. When the cells are exposed to an agonist of the receptor, intracellular calcium release results in the activation of aequorin, and the resulting light emission is recorded in a microplate luminometer.

As some receptors display constitutive activity, or other unknown characteristics that result in the counterselection of cell lines expressing them, we have expressed a dozen of these troublesome receptors in 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.

The orphan 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. 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 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.

4. 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 ascetic 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).

5. 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 TNFα 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).

6. 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 that contributes 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 (Ledent et al. Nature 388: 674-678, 1997). Additional experiments were made in collaboration with various groups, in order to delineate further the role of adenosine receptors in various aspects of physiology.

We have shown previously that the CB_1 cannabinoid receptor is involved in the development of opioid dependence. We have investigate 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).

The role of the adenosine A_{2a} receptor in the hypnotic effects of ethanol was assessed in mice. 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).

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

We had previously generated a knockout model for the CB_1 receptor, the central receptor for the active compounds of *Cannabis*, and for the endogenous cannabinoid anandamide (Ledent et al. Science 285 : 401-404, 1999). This model was further tested in collaboration with a number of groups.

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). 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 studies have suggested that the endocannabinoid system may play a role in the reinforcing effects of ethanol. Disruption of CB_1 receptors in mice 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, 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 doses. The severity of alcohol withdrawal-induced convulsions was also increased in $CB_1^{-/-}$ mice. These results suggest that CB_1 receptors participate in the regulation of ethanol drinking and demonstrate that their disruption lead to increased ethanol sensitivity and withdrawal severity (Naassila et al. 2003).

8. Structure-function of CCR5 and chemokines

The manuscripts describing that transmembrane helices 2 and 3 of CCR5 contain important structural elements for the activation mechanism of chemokine receptors, and that mutation of these domains affect differently the binding of MIP-1α and RANTES have been published

(Govaerts et al. 2003, Blanpain et al. 2003). We have started to analyze the dimerisation states of CCR5 and other related receptors, and these studies are presently going on. 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).

9. Other receptors

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).

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Study of the mechanisms underlying vestibular compensation, a model of brain plasticity.

During last year, our laboratory continued to tackle three projects. The first was vestibular compensation, the second concerned perturbations of synaptic plasticity in a transgenic mice model of Alzheimer's disease while the domain of the third study was memory and long-term potentiation.

I. VESTIBULAR COMPENSATION

Background

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

Experiments on brainstem slices

We investigated the properties of the vestibular neurons in brainstem slices, using intracellular microelectrodes. Responses to currents injected intracellularly in vestibular neurons from control animals were compared to those obtained in vestibular neurons from animals labyrinthectomized one week earlier. Using this technique during year 2002, we investigated the pacemaker currents in the vestibular neurons. We have found that one of them, the low-threshold calcium current, was increased 7 days after labyrinthectomy at a moment when recovery of spontaneous discharge is achieved. Using antibodies against the 3 species of low-threshold calcium channels (α1G, α1H and α1I), we have shown that the observed increase in calcium current was not related to an increase synthesis of these channels. This research was published this year in *NeuroReport*. In a work carried out in collaboration with the CNRS laboratory headed by P.P. VIDAL (Paris), it was demonstrated that this modification is maintained and even strengthened in the long run (1 month after the lesion) (*Journal of Neurophysiology, 2003*).

We have thus demonstrated that adult vestibular neurons deprived of one of their major synaptic inputs undergo modifications in their electroreceptors. It is the first time that this type of plasticity is demonstrated to occur in adult animals, in vivo. In our model, the strenghtening of pacemaker channels allow the vestibular neurons to develop more spontaneous activity a few days after labyrinthectomy. This phenomenon accounts at least partly for the recovery of spontaneous activity in every neuron of the vestibular nucleus 7 days after destruction of the labyrinth.

II. SYNAPTIC PLASTICITY IN ALZHEIMER'S DISEASE

For a few 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. On one hand, it is well known that patients suffering from Alzheimer's disease are afflicted with severe memory deficits. On the other hand, there is now general agreement among the scientific community that memories are underlied by changes in synaptic strength. We therefore decided to study the long-term potentiation (LTP) of the synaptic strength induced by high frequency stimulation of the presynaptic fibers in hippocampal slices of transgenic mice related to Alzheimer's disease. In 1999, we discovered that LTP was impaired in mice carrying a mutation of the amyloid precursor protein (APP) (Moechars et al., *The Journal of Biological Chemistry*, 1999, 274, 6483-6492).

Aβ amyloid peptide (especially 1-42) is believed to play a crucial role in the pathogenesis of AD. Normaly, a protein anchored in the membrane (APP) is cleaved essentially by an α-secretase (probably ADAM 10) in the middle of the sequence of the Aβ peptide. In an alternative catabolitic pathway, APP cleaving by two other enzymes (β and γ secretases) yields Aβ amyloid peptide. To hindrance Aβ peptide formation, the general target is to search for inhibitors of γ secretase. The laboratory of Prof. Fahrenholz at University of Mainz in Germany, with which we collaborate, used a different strategy. It overexpressed α secretase (ADAM 10) in a transgenic mice model of Alzheimer Disease. We found that this treatment corrected the deficit in the LTP observed in our transgenic model of AD (in course of revision in the Journal of Clinical Investigation).

III. MEMORY AND LONG-TERM POTENTIATION

We are interested in the mechanisms of the LTP which can be induced in hippocampal slices in synapses between Schaffer's collaterals and CA1 hippocampal neurons. The mechanisms underlying the early phase (1 h) of LTP (early-LTP) and those underlying the late phase (4 h) of LTP are different. The first are well known. The second are not. Early LTP is generally triggered by 1 train of high frequency stimulation (100 Hz, 1 sec) while late LTP is generally induced by 4 trains delivered every other 5 min. An intermediate long-lasting LTP can be induced by two trains separated by 20 sec.

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 while this laboratory was involved in assessing the role of Ca^{++}/Calmodulin Kinase Kinase β (CaMKKβ) in memory. For the three months preceeding her stay in London and during a short stay in Belgium in July 2003, Laurence RIS assessed LTP in CaMKKβ-knocked out mice. It was so found that Ca^{++}/Calmodulin Kinase Kinase was important for LTP induced by 4 trains and for some, but not all, types of hippocampus-dependent long-term memory. These results were published in *The Journal of Neuroscience*.

As forementioned, we are interested in the 4 h - LTP whose mechanisms are largely unknown. However, to induce and record a reliable LTP for 4 h is by far more difficult than to monitor it

for 1 h. Brigitte CAPRON succeeded to do it. We are now able to monitor late - LTP (4 h) in routine. Brigitte CAPRON also succeeded to perform protein 2D-electrophoresis on samples of the CA1 region taken from slices submitted to a chemical treatment inducing a late-LTP.

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Progress Report of the Research Group of

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I. Physiology and physiopathology of the basal ganglia system

Our results on the modulation of the glutamatergic corticostriatal transmission and synaptic plasticity in the accumbens nucleus and on the modifications of gene expression induced by adenosine receptors have been published (1).

Following these studies on the modulation of synaptic plasticity, we have developed a quantitative molecular model allowing to reproduce the modifications of synaptic efficacy as they are experimentally observed during long term potentiation (LTP) and long term depression (LTD) (5). This approach allowed us to propose intracellular signalling pathways initiated by different increases in intracellular calcium concentration which should be responsible for the induction of LTP or LTD, respectively (5).

We have developed an experimental model of Huntington's disease in rat or mouse by using subchronic injection of 3-nitropropionic acid (3NP) and demonstrated the high reproducibility of this model in terms of lesion. The high reproductibility of this 3NP model allows studies of neuroprotection. In this line, we have studied the potential neuropotective effects of adenosine A_{2A} receptors by using transgenic mice deficient in A_{2A} receptors and selective A_{2A} antagonists or agonists ligands (2,3,4). Our results demonstrated that the 3-NP induced lesion depends on a balance between the deleterious activity of presynaptic A_{2A} receptors and the neuroprotective activity of postsynaptic A_{2A} receptors. This suggests that thereapeutic use of A_{2A} antagonists in Huntington's disease as in other neurodegenerative diseases, could present biphasic "neuroprotective-"neurotoxic" effects depending on the used dosages (2,3,4). 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 (9).

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 (8). 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 (8) that could be relevant to the understanding of the selective neuronal death in Huntington's disease.

We have pursued the construction of transgenic mice allowing the study of the specific roles of striatopallidal or striatonigral neurons.. We had obtained mice strains expressing the CRE recombinase under the control of the A_{2A} receptor promotor inserted in a BAC (bacterial artificial chromosome). These lines have been crossed with a reporter strain (Rosa26) in order to determine whether they selectively expressed CRE in striatopallidal neurons. Different experimental approaches (immunohiostochemistry, histochemistry, intracerebroventricular injection of colchicine, …) allowed to demonstrate that the CRE expression is indeed mostly restricted to striatopallidal neurons. We will now start crossing these mice with strains of mice allowing the selective inactivation of genes in this population of neurons.

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

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

We have characterized cellular mechanisms leading to the alterations observed in calretinin-deficient mice through an approach combining electrophysiology in vitro (patch clamp in the preforated patch configuration) of granular cells of the cerebellum and computer modeling (6). We demonstrated that the absence of calcium buffering modifies the intrinsic excitability through a modification of the response of calcium-activated potassium channels (6)

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.

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 (7). We suggested that this oscillation was generated by Purkinje cells whose behavior became rythmic and synchronous in these mice. 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 functionnal roles (7).

III. Molecular characterization of the gastrointestinal pacemaker mechanism.

We studied transgenic mice SK3 tTA, carriers of the conditionnal inactivation of the "calcium activated potassium channel" SK3, (Prof. Adelman, Portland). The apamine-sensitive SK3 channel is expressed in gut exclusively by one type of interstitial cells: "fibroblast-like cells (FLC)", that we have previously identify in the smooth muscle. 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. 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.

In line with our previous studies, the distribution of the interstitial cells of Cajal (ICC), identified by their immunoreactivity for the receptor tyrosine kinase KIT (KIT+ ICC), was characterized in several human disorders of the gastrointestina transit. We have finalized our study on the pathology of the cardia in Allgrove's (3A) syndrome (11). Other diseases and a quantitative pathological approach are currently being considered.

FLC expressing SK3 channel are therefore a new functional type of cells, besides interstitial cells of Cajal (ICC), modulating the excitability of gut smooth muscle and its response to the stimulations of the enteric nervous system. Studies are running to precise development of FLC SK3+, their functional coupling to ICC KIT+ and smooth muscle and to analyze their properties in human and gut motility diseases. We believe that these studies may open new concepts on the control of gut motility and its diseases (12).

We have described the gut distribution of the purinergic receptor P2X7. Indeed, purinergic neurotransmission (ATP) is a major inhibitory component of the response of the smooth muscle of the digestive tract but there was no available data on the localisation of this receptor. The P2X7 receptor is present in nerve terminals and in glial cells but is not expressed by ICC or FLC (9).

The search for genes involved in the pacemaker function of ICC has been initiated in the laboratory. Substractive PCR experiments have been performed in order to identify genes expressed in the normal muse jejunum but absent in the jejunum of ICC deficient (W^{lacZ}/W^{v}) mice. 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 a gene and its product which are specifically expressed in ICCs. Functional experiments using pharmacological tools and transgenic knock-out mice are in progress.

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ALZHEIMER'S DISEASE AND RELATED DISORDERS

Alzheimer's disease (AD) is the most frequent form of 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 play an important role in the AD pathophysiology. To date, 3 causal 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*) (Rademakers et al. 2003a). More than 150 different mutations have been reported in >320 families worldwide ("Alzheimer Disease Mutation Database": http://molgen-www.uia.ac.be/ADMutations). Multiple studies have shown that the 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 ranging from pure forms of AD to pure forms of other dementias, of which the most common is frontotemporal dementia (FTD). Recent studies have shown that 10-43% of all familial FTD cases are associated with mutations in the gene encoding the microtubule associated protein tau (*MAPT*). Because of the substantial clinical overlap and phenotypic heterogeneity in these autosomal dominant dementias, careful neuropathological brain examination is crucial in establishing a definite diagnosis. For AD, both Aß plaques and tau-positive neurofibrillary tangles are a prerequisite for the neuropathological diagnosis of AD. FTD can be pathologically classified into three different categories: FTD with neuronal and glial tau depositions, FTD with ubiquitin-positive inclusions and FTD with neuronal loss and spongiosis but without intracellular inclusions. Clinically there are no features that can reliably distinguish between these three groups.

Identification of mutations in known dementia genes

We reported a 6-generation Belgian family with autosomal dominant early-onset dementia segregating the R406W in MAPT, and in 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., 2003b).

We identified a novel missense mutation (V715A) near the γ-secretase cleavage site of APP in a German patient with presenile AD. 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 lead to a 4.1 times increased Aß42 to Aß40 ratio in HEK293 cells (Cruts et al., 2003).

Identification of susceptibility genes for dementia

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., 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. 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., 2003).

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PERIPHERAL NEUROPATHIES

Charcot-Marie-Tooth (CMT) disease is the most common inherited disorder of the peripheral nervous system, with an estimated frequency of 1 in 2500 individuals. At present, mutations resulting in CMT have been reported in 26 genes (http://molgen-www.uia.ac.be/CMTMutations/). In the past year we successfully identified 2 novel loci and 3 novel genes for Charcot-Marie-Tooth (CMT) neuropathies. We also performed genotype-phenotype correlations for known genes for CMT and related inherited peripheral neuropathies.

Identification of novel genes

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 nerve-conduction velocities was discovered. Reduced nerve conduction velocities (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. In a collaborative effort with Danny Huylebroeck and colleagues from the Department of Developmental Biology (K.U.Leuven), 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. 2003a).

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. Hereditary sensory neuropathy type I (HSN-I) mapped to chromosome 9q22.1-q22.3 and was caused by mutations in the *serine palmitoyl-transferase subunit 1 (SPTLC1)* gene. Charcot-Marie-Tooth type 2B (CMT2B) is a hereditary motor and sensory neuropathy assigned to chromosome 3q13-q22. We now found two missense mutations in the small GTPase late endosomal protein gene RAB7, associated with the CMT2B phenotype in distinct families (Verhoeven et al. 2003b). 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 type 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. 2003b).

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. 2003).

Identification of novel chromosomal loci

Dominant intermediate Charcot-Marie-Tooth (DI-CMT) is a genetic and phenotypic variant of classical CMT neuropathy, 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 (Jordanova et al. 2003a). This is the third locus reported for DI-CMT.

Identification of novel mutations in known genes

In order to perform genotype-phenotype correlations we screened two genes causing dominant or recessive types of CMT: neurofilament light chain polypeptide (*NEFL*), and ganglioside-induced differentiation-associated protein 1 gene (*GDAP1*). Mutations in *NEFL* were recently 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 six 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. 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. 2003b). 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 (Ammar et al. 2003).

Therapeutic approaches in peripheral neuropathies

Finally, we commented on a the first therapeutic tool for the most common variant of CMT, the CMT1A duplication on chromosome 17p11.1-p12 (De Jonghe and Timmerman 2003).

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EPILEPSIES

The idiopathic epilepsies are complex disorders resulting from interplay between genetic and environmental factors. Although a positive family history is often present, the exact inheritance pattern is usually not clear. However some idiopathic epilepsy syndromes have a more prominent monogenic component. In these syndromes and in some exceptional large families molecular genetic studies have made major progress. So far, 35 loci and 14 genes have been identified, illustrating the enormous genetic heterogeneity.

Mutations in known genes

We previously reported mutations in *SCN1A* in severe myoclonic epilepsy of infancy (SMEI) a rare disorder occurring in isolated patients. In the initial study we mainly detected *de novo* heterozygous nonsense mutations, predicted to result in truncated or severely altered proteins. To further investigate the contribution of *SCN1A* mutations to the etiology 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. 2003). They also demonstrate that the nature and the localization of the mutations correlate with the phenotype.

We also performed a mutation analysis of *SCN1B* on 74 unrelated probands with generalized epilepsy with febrile seizures plus (GEFS+), febrile seizures (FS) or febrile seizures plus (FS+). In a family with FS+ and early-onset absence epilepsy, we identified a splice site mutation, which results in the deletion of 15 nucleotides at the 5'-end of exon 3. Our results provide further evidence that *SCN1B* mutations are associated with GEFS+. We also demonstrated a potential role for *SCN1B* mutations in the elicitation of absence seizures (Audenaert et al. 2003).

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- Claes,L., Ceulemans,B., Audenaert,D., Smets,K., Löfgren,A., Del-Favero,J., Ala-Mello,S., Basel-Vanagaite,L., Plecko,B., Raskin,S., Thiry,P., Wolf,N.I., Van Broeckhoven,C., De Jonghe,P.: De novo SCN1A mutations are a major cause of severe myoclonic epilepsy of infancy. Human Mutation 21: 615-621 (2003)
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SCIENTITIFIC ACTIVITIES 2003

Licentiate Theses

- **Karen Jennes**: 'Functionele analyse van genen verantwoordelijk voor dementie', Promotors: J. Theuns & C. Van Broeckhoven
- **Sylvia Lesseliers**: 'Identificatie en analyse van kandidaatgenen voor frontale kwab dementia gekoppeld aan chromosoom 17q21', Promotor: M. Cruts; Co-promotor: R. Rademakers
- **Arvid Suls**: 'Moleculair genetische analyse van erfelijke epilepsieën', Promotor: P. De Jonghe, Co-promotor: L. Claes

- **Bianca Van Broeck**: 'Transgene APPT714I muizen voor Alzheimer dementie', Promotors: S. Kumar-Singh & C. Van Broeckhoven; Begeleiders: M. Zabielski & K. Vennekens

MD Theses

- **Dominique Van Diest**: 'Klinische en moleculair genetische aspecten van erfelijke perifere zenuwziekten', Promotor: P. De Jonghe

Master Thesis

- **Julie van der Zee**: 'De ziekte van Alzheimer: Moleculaire Genetica', 'Alzheimer's Disease: Molecular Genetics', Promotors: M. Cruts & C. Van Broeckhoven

Graduation Reports

- **Linzy Berckmans**: 'Moleculair genetische analyse van idiopathische epilepsiesyndromen', Stagebegeleiders: L. Claes & D. Audenaert

FELLOWSHIPS AND AWARDS

Fellowships

- **Albena Jordanova**: NATO/Research Fellowship - Ministry of Foreign Affaires & Fund for Scientific Research 'Molecular characterization of inherited peripheral neuropaties and related disorders: a population based study', January 1 - December 31, 2004

Travel Awards

- **A. Jordanova**: FENS/IBRO to attend the International FENS/IBRO course 'Peripheral Nervous System - From Biology to Disease', Ofir, Portugal, June 30 - July 8, 2003
- **J. Theuns**: Fund for Scientific Research - Flanders (FWO-F) to attend the HGM 2003 Meeting, Cancun, Mexico, April 27-30, 2003
- **J. Theuns**: Fund for Scientific Research - Flanders (FWO-F) to attend the 1st European ESF Conference on Functional Genomics and Disease' Prague, Czech Republic, May 14-18, 2003

PRESENTATIONS AT MEETINGS

Invited Lectures

- **P. De Jonghe**: 'Genotype/phenotype relations in distal HMN. Frontiers in Neurodegeneration', ALS/MND meeting of the European ALS/MND group. Reisensburg, Germany, January 30 - February 1, 2003
- **P. De Jonghe**: Plenary Lecture. North American CMT Consortium Inaugural Meeting, London, Ontario, Canada, March 7-8, 2003
- **P. De Jonghe**: Recent advances in the molecular genetics of hereditary demyelinating neuropathies. 7th Congress of the European Federation of Neurological societies, Helsinki, Finland, August 30 - September 2, 2003
- **E. Nelis**: Molecular genetics of hereditary polyneuropathy. 9th Mediterranean meeting of child neurology, Dubrovnik - Croatia, May 29-31, 2003
- **J. Theuns/ C. Van Broeckhoven**: Molecular Genetics of inherited dementias. ESF Functional Genomics and disease 1st Conference, Prague, Czech Republic, May 14-18, 2003
- **V. Timmerman**: Inaugural lecture: 'Over zenuwcellen, stambomen en genen', University of Antwerp, Belgium, April 4, 2003
- **V. Timmerman**: 'La génétique et les progress récentes', Lay association meeting, Conférence CMT Belgique, Institut Lennox, Ottignies, June 14, 2003
- **V. Timmerman**: Invited speaker: 'Update on the Molecular Genetic research in Charcot-Marie-Tooth disease'. CMT Workshop at the University of Genova, Italy, November 7-8, 2003

- **V. Timmerman**: seminar: "Update on Molecular Genetic Research of Charcot-Marie-Tooth Neuropathies". Seminar organised by Odile deLapeyrière, INSERM U382 - IBDM, Campus de Luminy, University of Marseille, France, December 9, 2003
- **C. Van Broeckhoven**: 'Genetic contributions to Alzheimer's disease and related disorders'Galapagos, Mechelen, Belgium, April 16, 2003
- **C. Van Broeckhoven**: 'Het krimpende brein', Vlaamse Alzheimer Liga: Vierde Symposium voor familieleden van jong-dementerende, Beerse, May 10, 2003
- **C. Van Broeckhoven**: 'Genetics of Alzheimer dementia and related disorders','Alzheimer und andere primäre Demenzen', 73 Kongress der Deutschen Gesellschaft für Neurologie, Hamburg, Germany, September 3-6, 2003
- **C. Van Broeckhoven**: 'Dementie en erfelijkheid', Symposium 25 jarig Jubileum 'De Bijster', Wuustwezel, Belgium, November 22, 2003

Oral Presentations - Slide Sessions

- **P. De Jonghe**: Confirmation of linkage of autosomal dominant familial distal amyotrophy and spastic paraplegia (Silver Syndrome) to chromosome 11q12-14 and refinement of the candidate region, North American CMT Consortium Inaugural Meeting, London, Ontario, Canada, March 7-8, 2003
- **E. Nelis**: The Mutation Database of inhterited peripheral neuropathies. North American CMT Consortium Inaugural Meeting, London, Ontario, Canada, March 7-8, 2003
- **E. Nelis**: Mutations in GDAP1 are associated with axonal and demyelinating CMT. North American CMT Consortium Inaugural Meeting, London, Ontario, Canada, March 7-8, 2003
- **R. Rademakers**: MAPT R406W mutations arose independently in 3 families from Western Europe. 6th International Conference AD/PD, Seville, Spain, May 8-12, 2003
- **J. Theuns**: Alzheimer-associated promotor polymorphism -22C>T causes a critical neuron-specific decrease of presenilin 1 expression. HGM 2003 Meeting, Cancun, Mexico, April 27-30, 2003
- **V. Timmerman**: Molecular genetics of Charcot-Marie-Tooth type 2B neuropathy (CMT2B), North American CMT Consortium Inaugural Meeting, London, Ontario, Canada, March 7-8, 2003
- **K. Verhoeven**: Mutations in the small GTP-ase late endosomal protein RAB7 cause Charcot-Marie-Tooth type 2B neuropathy (CMT2B). VIB Seminar, Blankenberge, Belgium, March 13-14, 2003
- **N. Verpoorten**: Identification of differentially expressed genes in motor- and sensory neurons. VIB Seminar, Blankenberge, Belgium, March 13-14, 2003

Poster Presentations

- **D. Audenaert**: A deletion in SCN1B is associated with febrile seizures and early-onset absence epilepsy. VIB Seminar, Blankenberge, Belgium, March 13-14, 2003
- **K. Coen**: Genetic linkage and refinement of the Charcot-Marie-Tooth type 2B locus on 3q13-q22. VIB Seminar, Blankenberge, Belgium, March 13-14, 2003
- **L. Deprez**: Molecular genetics of the intermediate form of the Charcot-Marie-Tooth disease and the hereditary sensory and autonomic neuropathy type IV. VIB Seminar, Blankenberge, Belgium, March 13-14, 2003
- **I. Dierick**: Genetic linkage and mutation analysis in distal hereditary motor neuropathies. VIB Seminar, Blankenberge, Belgium, March 13-14, 2003
- **J. Irobi**: Confirmation of linkage of autosomal dominant familial distal amyotrophy and spastic paraplegia (Silver Syndrome) to chromosome 11q12-14 and refinement of the candidate region. VIB Seminar, Blankenberge, Belgium, March 13-14, 2003

- **A. Jordanova**: Mutations in the neurofilament light chain gene (NEFL) cause early onset severe Charcot-Marie-Tooth disease. VIB Seminar, Blankenberge, Belgium, March 13-14, 2003
- **E. Nelis**: Mutations in GDAP1 are associated with autosomal recessive Charcot-Marie-Tooth disease with demyelination and axonopathy. VIB Seminar, Blankenberge, Belgium, March 13-14, 2003
- **R. Rademakers**: Mutation Analysis of candidate genes for chromosome 17-linked tau negative FTD. 4th International Conference on Fronto Temporal Dementias, Lund, Sweden, April 24-26, 2003
- **J. Theuns**: Alzheimer-associated c-allele of the promoter polymorphism -22C>T causes a critical neuron-specific decrease of presenilin 1 expression. ESF Functional Genomics and disease 1st Conference, Prague,
- **K. Verhoeven**: Mutations in the small GTP-ase late endosomal protein RAB7 cause Carcot-Marie-Tooth type 2B neuropathy (CMT2B). FENS Summer school, Ofir, Portugal, June 30 - July 7, 2003

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Functional analysis of a gene for progressive hearing impairment

Introduction

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

Research progress during the past year

1. The elucidation of the subcellular localization of DFNA5

The subcellular localization of DFNA5-GFP was cytoplasmic in HEK293T and in COS-1 cells. As the HEK293T and the COS-1 cell lines might not express the necessary partners, which consequently might lead to an incorrect cytoplasmic localization, we have transfected an etoposide-resistant and an etoposide-sensitive melanoma cell line (Lage et al., 2001). In these cell lines two populations of transfected cells were observed, 1 population with expression exclusively in the cytoplasm and one population with expression both in the nucleus and in the cytoplasm. These were preliminary results, further experiments need to be performed in the coming year.

2. Additional families with DFNA5 mutations and the formulation of a gain-of-function hypothesis

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 harboring 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. Although at the genomic DNA level, the mutations in DFNA5 leading to hearing loss are diverse, at the mRNA level all these mutations lead to exon 8 skipping, indicating that only this particular event might cause hearing impairment. 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 gained very recently. It was demonstrated that human mutant DFNA5 was toxic for yeast (Figure 1) (Gregan et al., 2003) as well as for mammalian (Figure 2) (Van Laer et al., in press) cells.

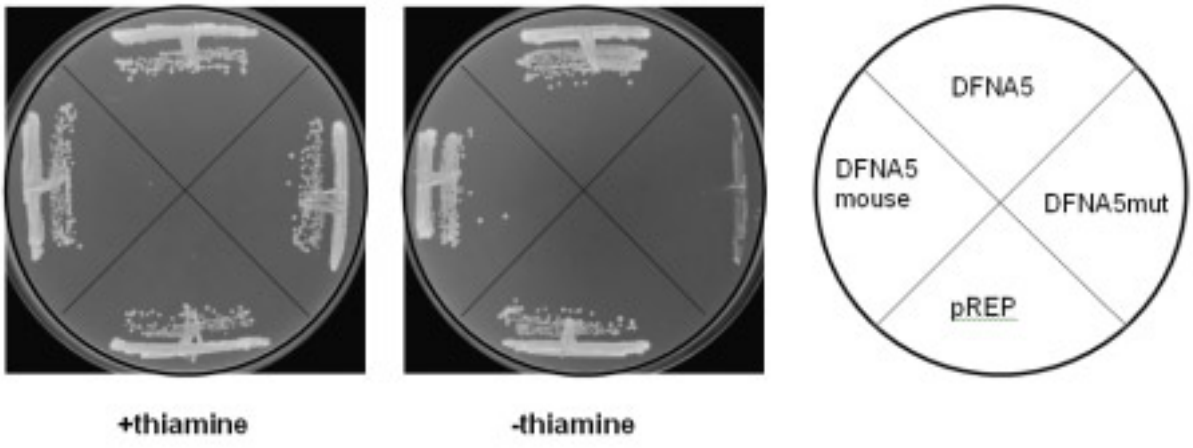

Figure 1. Heterologous expression of DFNA5 proteins in yeast. Wild-type strain (P138) transformed with either empty vector pREP3x (pREP), or a construct containing mouse Dfna5h (DFNA5mouse), human wild-type DFNA5 (DFNA5) or the human mutant allele (DFNA5mut) were grown on EMM plates with or without added thiamine.

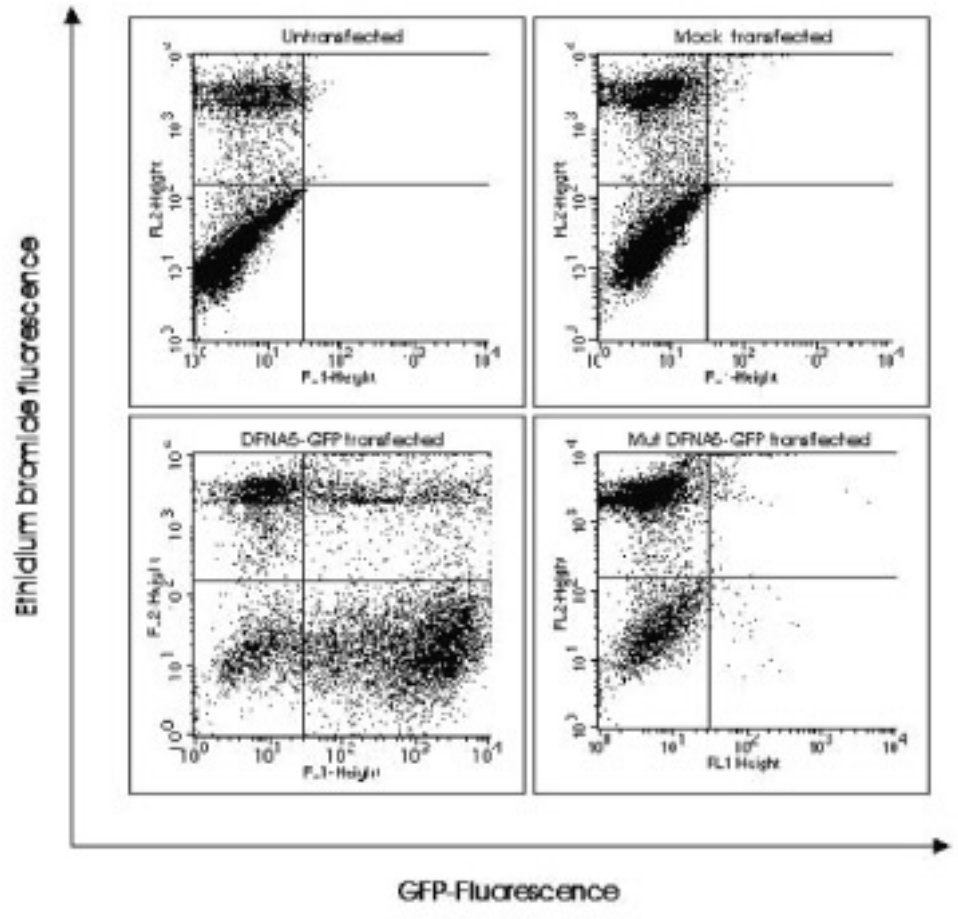

Figure 2. Transfection experiments in HEK293T cells. Twenty-four hours post-transfection, HEK293T cells were trypsinized and studied in the presence of ethidium bromide using FACScan flow cytometry. In the dot plots, the lower left quadrant represents viable GFP^- cells, the lower right viable GFP^+ cells, the upper left dead GFP^- cells, and the upper right dead GFP^+ cells. Similar results were obtained using COS-1 cells (not shown).

3. Characterization of a DFNA5 knockout mouse

During the past year, the replacement of the genetic background of the DFNA5 mouse into the C57Bl/6J and CBA/Ca genetic background was completed. Homozygous mice from the 10th generation, in which 99.9 % of the genetic material is derived either from the C57Bl/6J or CBA/Ca strain, are available. Frequency-specific ABR (Auditory-evoked Brain Response) tests were continued. Mice from the CBA/Ca genetic background aged between 12 and 15 months and between 17 and 19 months were tested. Unfortunately, even at an age of approximately 1.5 years no obvious hearing impairment was observed in DFNA5 knockout mice. Because no significant differences were detected in vestibular function between DFNA5 knockout mice and their wild-type littermates from the C57Bl/6J genetic background (see report 2002), and in view of the results obtained 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 knockout mice and their wild-type littermates at the level of the outer hair cells of the apical turn. 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, be it with opposite effects: DFNA5-/- mice show an increased (C57Bl) versus a decreased (CBA/Ca) number of fourth row outer hair cells when compared to wild-type littermates (Figure 3).

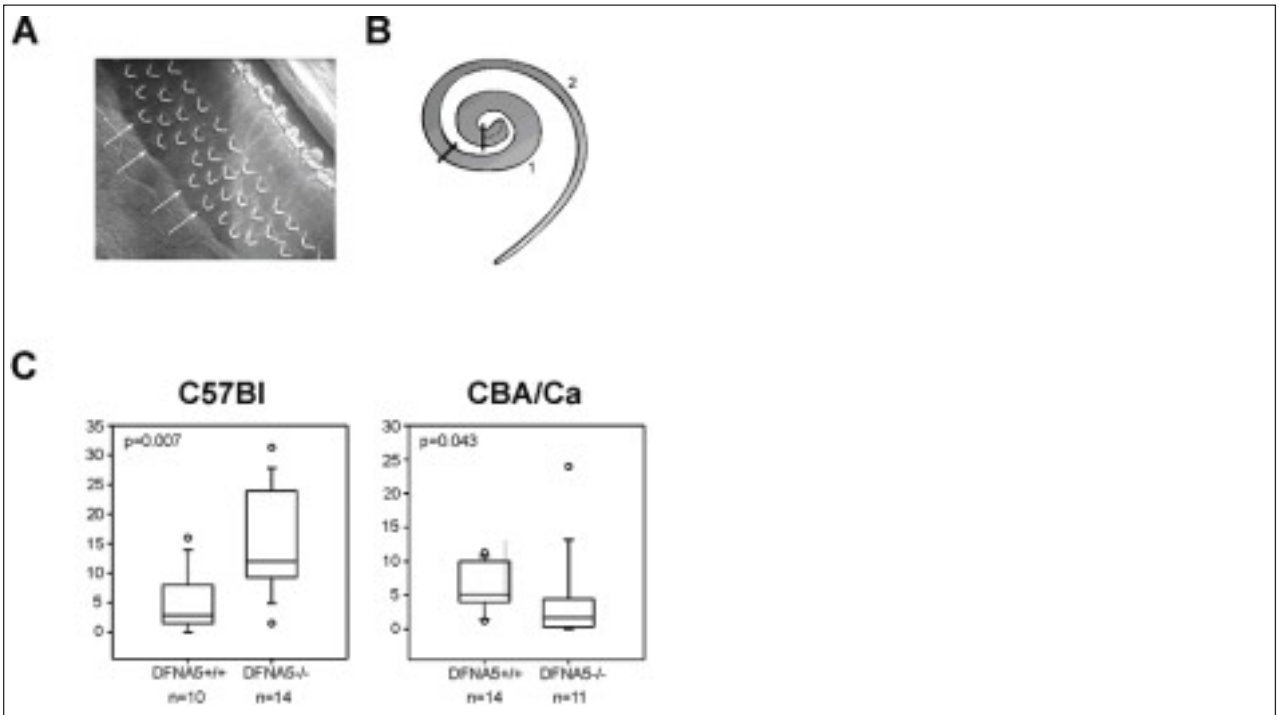

Figure 3. Scanning electron microscopy of cochlear hair cells in the organ of Corti. A typical result is presented in panel (A). The arrows indicate supernumerary (fourth row) outer hair cells, which were counted along the cochlear turns. (B) A diagram representing the division of the cochlea, for counting 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. In addition, the 10% to 90% percentiles are given by the thin lines. All values outside the 10% to 90% percentiles are represented by small circles.

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 (Figure 4).

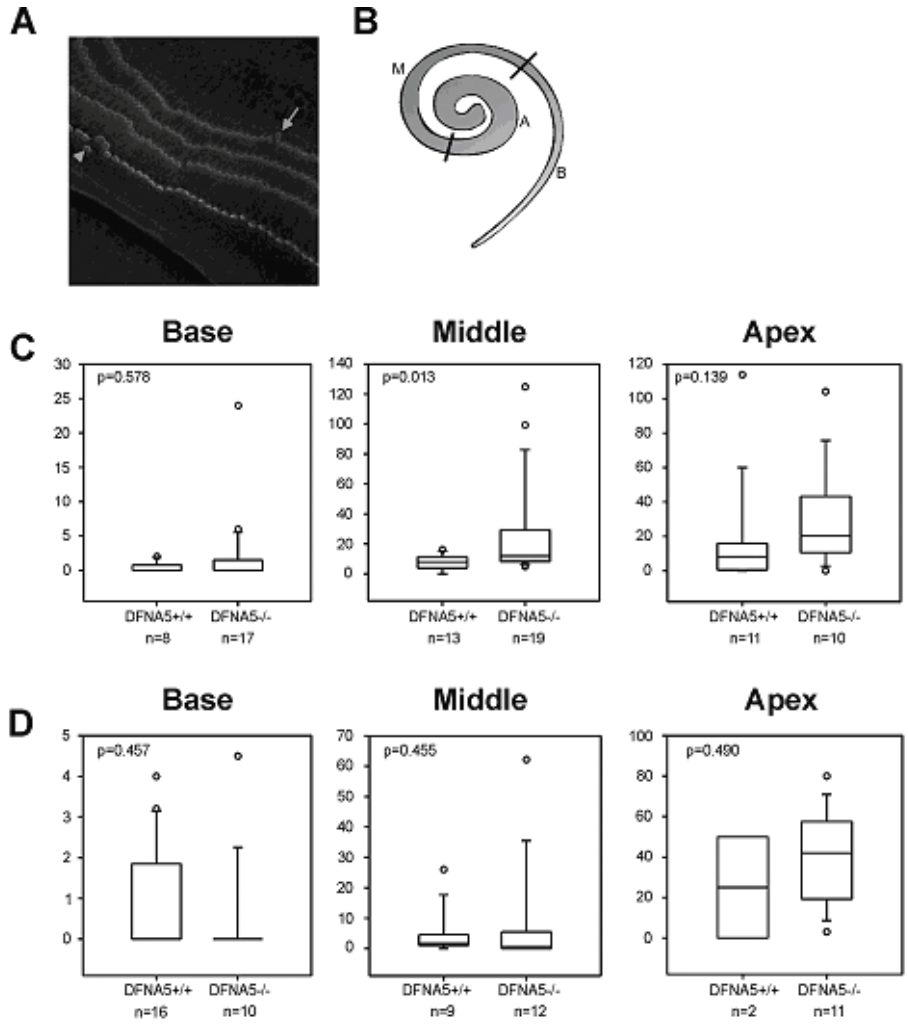

Figure 4. Organ of Corti explants. A typical result is shown in panel (A). The arrow indicates a fourth row outer hair cell, while the arrowhead indicates a supernumerary inner hair cell. (B) A diagram representing the division of the cochlea, for counting purposes, in an apical (A), a middle (M) and a basal (B) portion. (C-D) Counts of fourth row outer hair cells in 3 cochlear portions for DFNA5-/- mice and their wild-type littermates, in C57Bl (panel C) and CBA/Ca (panel D) genetic background. Box plots have a similar structure as described in the legend to figure 3.

A complete histopathological analysis of 46 other organs, performed by a specialised company Frimorfo (Fribourg, Switzerland), revealed no additional abnormalities in the DFNA5 knockout mice. A manuscript describing the results on the DFNA5 knockout mouse is in preparation (Van Laer et al., in prep)

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

1. Functional magnetic resonance study of the network for associative semantics in the intact brain

In temporal cortex near air cavities fMRI images suffer signal loss due to susceptibility artefacts. We adapted our fMRI acquisition protocol and minimized these artefacts by combining an 8-channel receive only head coil and the GRAPPA parallel imaging technique which is less sensitive to susceptibility artefacts in the sagittal plain (collaboration with the Radiology Department, P. Van Hecke). In a recent fMRI study in 19 subjects aged between 55 and 75 years we confirmed the composition of a distributed left-hemispheric system that is recruited when subjects perform associative-semantic judgements compared to visuoperceptual discriminations regardless of input modality, written words or pictures (Vandenbulcke et al., 2003). In the associative-semantic condition we presented three stimuli, one on top, two at the bottom. Subjects had to indicate which test stimulus matched the sample stimulus

most closely in meaning. The stimuli consisted of either words or pictures. In the visuoperceptual control task we presented three stimuli, one on top and two at the bottom. Subjects had to press a left- or right-hand key depending on which test stimulus matched the sample stimulus most closely in size on the screen. The activated distributed system consisted of left inferior temporal and anterior temporal regions, superior temporal sulcus and posterior middle temporal g., inferior frontal g. and inferior frontal s. The system was activated regardless of input modality, words or pictures, replicating a previous positron emission tomography (PET) study (Vandenberghe et al., 1996). As a shorthand, we will refer to this system below as the "common semantic system". This does not imply that the functions of each of its components are related to associative semantics. One purpose of the current proposal is precisely to dissect these funtions.

In addition to the original activation pattern (Vandenberghe et al., 1996), the right mid-fusiform g. was more active when subjects performed an associative-semantic task with pictures than when they performed a visuoperceptual task. This activation was not present when the input consisted of words (interaction between modality and task) (Vandenbulcke et al., 2003). The homologous left-sided area was activated when the two tasks were compared for both words and pictures.

We recently performed a detailed anatomical and cognitive analysis in a patient who suffered a lesion confined to the right mid-fusiform region. The patient performed the associative semantic task within the normal range but manifested striking impairments on tasks of object identification (such as pseudo-object decision), drawing from memory and verbal associative semantic tasks with highly imageable concepts (Vandenberghe et al., 2003)). We are currently testing the patient for knowledge of perceptual and functional-associative attributes of large number of exemplars taken from a range of semantic categories (collaboration with the

Department of Psychology).

The patient's cognitive profile together with the fMRI findings in healthy controls lead us to the hypothesis that the right midfusiform g. is involved in mapping a detailed perceptual representation onto a fine-grained structural description of an object and vice versa (two-way mapping between a perceptual representation and a stored representation). We propose that the stored representation is subsequently transferred to the left-sided anterior fusiform area, the first relay station related to lexical-semantic representations. When input consists of

written words, a detailed perceptual representation is mapped onto the visual word form in left midfusiform g. and from there to the anterior fusiform g., the first station in the word processing pathway related to lexical-semantic representations. From the left anterior fusiform area and the inferior temporal g. onwards the distributed left-hemispheric system is shared regardless of input modality. Anterior temporal cortex mediates combinatorial associations (Vandenberghe et al., 2002) between distributed cortical representations (Tyler and Moss, 2001), comparable to the associative role of the entorhinal cortex and hippocampus in episodic memory. In an alternative model there is no processing beyond a pre-lexical or structural description level in ventral occipital or inferior temporal regions. Instead, the lexical-semantic processing pathway goes via the middle temporal g. to more anterior temporal and frontal areas (Chertkow et al., 1997; Whatmouth et al. 2002).

The cognitive distinctions between structural descriptions, lexical representations and associative semantics in our model are in agreement with the Hierarchical Interactive Theory of object processing (Humphreys and Forde, 2001). This theory also features feedback and parallel activation between hierarchically later and earlier stages. The distinctions between written word form and lexical-semantic representations fits in the framework of the Independent Network model (Caramazza, 1997). This model distinguishes between a lexical-semantic network and orthographic and phonological input and output lexemes (Caramazza, 1997).

2. Associative semantics, ageing and cortical neurodegeneration

A strategy to further dissect the functions of the constituents of the "common semantic system" is to study the system in different populations. In cortical neurodegenerative disease, how do verbal and semantic dysfunction, changes in large-scale cognitive brain systems, and neurochemical pathogenetic processes relate to each other?

During the past year we have focussed on three populations

1. Healthy elderly controls. One of the most frequent age-related changes consists of episodic memory decline while semantic memory measures remain relatively intact.
2. Patients with mild cognitive impairment (MCI) (Petersen, 2003) who are naive to cholinesterase-inhibitors. In MCI the predominant cognitive deficit is an episodic memory deficit while other cognitive domains are clinically intact. The conversion rate to clinically probable Alzheimer's disease is 15% per year (Petersen, 2003). MCI partially overlaps with the entity of "incipient" Alzheimer's disease.

3. Patients with primary progressive aphasia (PPA). PPA is a cortical neurodegenerative disorder characterized by predominance of language deficits (e.g. word finding), while other domains, such as non-verbal episodic memory, are relatively intact, at least at the initial stage (Mesulam, 2003). The majority of cases are due to frontotemporal degeneration (Mesulam, 2003). We have included both patients with non-fluent progressive aphasia and with semantic dementia (Hodges and Miller, 2001; Mesulam et al, 2003).

We are investigating the relationship between three different levels: Changes in cognition, changes in fMRI brain activity patterns and pathogenetic processes at a neurochemical level, i.e. cholinergic depletion. Contrary to the classical cholinergic hypothesis in Alzheimer's disease (Perry et al., 1978), postmortem studies of patients diagnosed with mild cognitive impairment or early-stage ProAD have questioned the role of cholinergic depletion in the early disease phase (Davis et al., 1999; DeKosky et al., 2002). The functional reorganisation of large-scale cognitive brain systems in response to brain injury forms a crucial intermediary

step between pathogenetic processes and cognitive manifestations and may help us resolve the discrepancies between neuropathological data and clinical manifestations. This will enhance our understanding of the transitions between ageing and MCI and between MCI and early ProAD.

Our measure of cholinergic status is N-[11C]Methylpiperidin-4-yl Propionate (PMP). PMP is a substrate for acetyl- and butyrylcholinesterase. These enzymes completely hydrolyse PMP to N-[11C]Methyl-4-piperidinol, a hydrophilic product that does not cross the blood-brain barrier and is trapped locally in the brain (Iyo et al., 1997; Kilbourn et al., 1998; Koeppe et al., 1999). In mild to moderate ProAD (Kuhl et al., 1999; Herholz et al., 2004) PMP levels are decreased compared to healthy controls in neocortical regions and amygdala but not in the basal nucleus of Meynert (Herholz et al., 2004).

Research questions

1. In healthy ageing and in MCI, does the common semantic network change despite the relative intactness of naming and semantic memory performance? Is there a measurable cholinergic depletion in MCI compared to cognitively intact controls and does it relate to the cognitive and fMRI activity patterns?

2. In PPA, do the changes of the common semantic system differ depending on input modality and depending on the neurolinguistic profile of these patients (i.e. the distinction between semantic dementia and nonfluent progressive aphasia)? Does a cholinergic depletion play a role in the naming and semantic memory deficits in PPA?

Methods

We have recruited patients via the Memory Clinic of the Department of Neurology, University Hospital Gasthuisberg, Leuven, which is headed by the applicant. Participants undergo detailed neuropsychological examination of non-verbally mediated executive

functions (Raven's Colored Progressive Matrices), verbal (Auditory Verbal Learning Test and Wechsler Memory Scale Logical Memory) and non-verbal episodic memory (Rey Visual Learning test), visuoperceptual tests (Birmingham Object Recognition Battery), and language testing (validated Dutch version of the Aachen Aphasie Test (AAT) and semantic associative task of the Psycholinguistic Assessment of Language Processing in Aphasia (PALPA)). The neuropsychological data are analysed using Factor Analysis.

Subjects participate in an epoch-based fMRI experiment using the same associative-semantic and visuoperceptual conditions described in section 1. For random-effects comparisons between groups we need at least 14-20 subjects per group.

As for the PMP measures, we are currently able to create and compare parametric images of kinetic constant k3 between subjects. We have studied 8 healthy controls, 6 MCI patients and 6 PPA. We will develop a method to correct for atrophy and partial volume effects aready at the image reconstruction stage using a new method that segments the image on the basis of MRI and PET characteristics into grey matter, white matter and CSF (collaboration with the Nuclear Medicine Department, P. Dupont) (Baete et al., 2004).

Recent findings

A fixed-effects analysis of 9 healthy individuals aged between 55 and 65 years and 9 individuals aged between 65 and 75 years revealed that the younger group showed significantly stronger left prefrontal activation than the older group. In right prefrontal cortex the pattern was opposite: Higher activity in the elderly compared to the younger group. This age-related decrease of the left-right prefrontal gradient in semantic memory tasks is similar

to what has been described in ageing studies of episodic memory (Cabeza et al., 1997) and working memory (Reuter-Lorenz et al., 2000). We want to confirm these findings in a larger number of subjects, including also an age group between 75 and 85 years. This cohort of elderly subjects will undergo detailed yearly neuropsychological testing. Longitudinal changes in the fMRI patterns measured every 2 year will be correlated with neuropsychological changes within subjects.

A fixed-effects comparison between 11 MCI patients and 11 cognitively intact elderly controls revealed no change in performance on the associative-semantic task (as would be expected given the definition of MCI). Neuroanatomically, however, the left posterior superior temporal s. and middle temporal g. were significantly less active than in healthy controls, both for pictures and words. Previous patient lesion and functional imaging studies (Vandenberghe et al., 1996; Chertkow et al., 1997; Whatmouth et al., 2002) have implicated this area as a core structure of the common semantic system. These data demonstrate the value of fMRI in revealing changes in brain processes at a stage when they are still below the threshold of clinical or standard neuropsychological expression. Follow-up neuropsychological and psychophysical measures will reveal which emerging cognitive dysfunctions correlate with the initial fMRI abnormalities.

A fixed-effects analysis in 9 patients with PPA (3 semantic dementia, 6 non-fluent progressive aphasia) indicate that, regardless of clinical classification, the left superior temporal s., the left inferior temporal g. and the left anterior temporal pole are less active than in normal controls. Performance on the naming subtest of the Aachen Aphasie Test correlated significantly with fMRI activity levels in the left anterior fusiform area, further corroborating our hypothesis that this area is involved in activating the lexical-semantic representations that correspond to word or picture input (see Section 1 Cognitive model).

A factor analysis of the neuropsychological scores of the entire study group (11 MCI, 9 PPA and 18 cognitively intact matched controls) revealed 3 significant latent variables (factors):First, AAT naming, AAT comprehension and the PALPA semantic association test; second, delayed recall and recognition tasks; and third, executive functions and AAT spontaneous speech performance. A random-effects linear regression analysis revealed that the first factor correlated significantly and positively with activity in the left anterior temporal pole (regression coefficient 0.65) and negatively with activity in the right anterior temporal pole (regression coefficient 0.71). The partial reversal of the left-right anterior temporal gradient in disorders of associative semantic memory is reminiscent of the pattern of changes of left-right prefrontal gradient in episodic memory (Cabeza et al., 1997; Grady et al., 2003). While however the right prefrontal activation correlated positively with performance (Grady et al., 2003), the right anterior temporal activation shows a negative correlation. Pathophysiologically, this may be analogous to the negative effect of non-dominant hemispheric perisylvian activations in post-stroke aphasia, suggesting either failing compensatory mechanisms or active disruption due to disinhibitory effects (Herholz and Heiss, 2000).

Conclusion

The following components are more or less finalised and ready to be reported:

1. picture-specific gate of entry into the common semantic system via the right mid-fusiform gyrus
2. left posterior superior temporal sulcus and middle temporal gyrus during associative semantic judgements in MCI
3. right anterior temporal cortex in primary progressive aphasia

These data have been presented or will be presented in 2004 at international meetings and will be submitted as a full paper. For the ageing study we will add 5-10 subjects above 75 years to confirm the age-related left-right prefrontal changes in semantic memory.

In healthy controls we are currently conducting an event-related fMRI study to examine the anatomical and functional relationship between picture-specific and word-specific processing in ventral occipitotemporal areas.

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Molecular and cellular Mechanisms of Development of Neuronal Connectivity in the Cerebral Cortex.

INTRODUCTION.

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

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

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

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

To address these issues we have focused on three distinct and complementary approaches :

1. In vivo study of the role of ephrin genes in cortical development using mouse transgenics.
2. In vitro study of the cellular effects of ephrins and other signaling pathways in cortical development using an organotypic culture system.
3. Identification of other genes involved in the patterning of cortical connectivity.

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 (5,6). In order to test for the effects of a more complete loss of ephrin function in the developing cortex, we have generated compound mutants for ephrin-A5 and for its receptors EphA4 and EphA7, expressed in complementary gradients in the thalamus.

Retrograde axon tracing analysis of these compound mutant mice first revealed a disruption of somatosensory thalamocortical topography, providing in vivo evidence that EphA4 and EphA7, together with their ligand ephrin-A5, act as thalamic axon guidance receptors that control topographic specificity within the somatosensory area (7). Altogether these results indicate that ephrin-A5 in the cortex acts as a graded repulsive cue for TC axons expressing graded levels of EphA receptors (including EphA4) to generate a precise topographic somatosensory map. Several questions remain concerning the cellular mechanisms of ephrin action in intra-areal mapping: do ephrins modulate axonal branching and/or pruning, much like in the retinotectal system, or do they rather control the guidance of thalamocortical axons as they invade the cortical plate? Analysis of the time-course of the defects found in ephrin/Eph mutants is now being done using anterograde tracing at several key developmental stages to try to clarify this question.

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 motor thalamus that normally only project to frontal motor areas start to project more caudally to the somatosensory areas. These results constitute the first direct evidence for the involvement of axon guidance factors in the generation of area-specific thalamocortical projections, and indicate that the same system of mapping label coordinates is used for the generation of topographic order of thalamocortical projections between and within individual cortical areas, suggesting an economical model of development of cortical connectivity (7).

Our data also point to a previously overlooked mechanism of generation of target-specificity in the developing brain. Indeed, using expression pattern analysis of ephrin/Eph genes in the early forebrain and anterograde axon tracing of mutant embryonic brains, we were able to show that ephrins were in fact controlling the initiation of target-specificity of cortical innervation through the early topographic sorting of thalamocortical axons in an intermediate target, the ventral telencephalon, which was further demonstrated by in vitro analyses (see section 2).

Importantly, the aberrant innervation of sensory areas by the motor system detected in the ephrin-A5/EphA4 mutants seems to have functional consequences: indeed, these mutants display a number of sensori-motor defects, including ataxic gait and paroxystic abnormal movements that could correspond to dystonia or seizures. we are currently analyzing the exact

nature of these defects using behavioural analysis and in vivo electrophysiological recordings.

In many aspects of developmental genetics, gain of function analyses constitute a useful complementary approach to loss of function. Therefore we have started to generate mice that display a gain of function of ephrin-A5, specifically in the developing cortex, using the cre/lox system transgenic technology, that allows to modify a locus in a temporally and spatially controlled way (8). Our previous studies have indeed revealed an interesting opportunity : several Eph receptor genes (in particular EphA7) are also expressed in gradients in the developing cortex, but their gradients of expression are inverted when compared to ephrin-A5 (our unpublished datas). Ectopic gradients of ephrin-A5 can thus be obtained by placing the ephrin-A5 coding sequence under the control of the regulatory sequences of the EphA7 gene.

This has now been achieved using a bacterial artificial chromosome (BAC) containing all the regulatory sequences of the EphA7 gene (previously isolated in the lab) where the ephrin-A5 coding sequence (flanked by a conditional lox/GFPstop/lox cassette) has been knocked in the first coding exon of the EphA7 gene by homologous recombination in E coli, followed by generation of the corresponding transgenic mice (9). Analysis of this line using in situ hybridization and immunohistochemistry for GFP has enabled to show that the transgene faithfully reproduces the endogenous graded expression patterns of the EphA7 gene in the cortex, thalamus, and midbrain (V. Depaepe, and P. Vanderhaeghen, unpublished data).

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

These various lines are now being analyzed with our previously used neuroanatomical techniques (quantitative histochemistry and axon tracing experiments (5,7)) to look for inversions and disruptions of various features of cortical development, focusing on the motor, somatosensory and visual systems. Preliminary data suggest that this approach will indeed reveal new functions of ephrins in the developing cortex, which are now being characterized in vitro and in vivo.

2. In vitro study of ephrins and other factors controlling the development of forebrain structures.

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

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

Taken together these in vitro results show that endogenous ephrin-A ligands distributed in the ventral telencephalon are required for the initiation of the topography of thalamocortical projections. This is fully consistent with the phenotypes described above in the ephrin/Eph mutant mice: in the absence of appropriate ephrin/Eph signaling, thalamic axons from the rostral thalamus growing from the presumptive motor thalamic nuclei, start to invade more caudal territories in the ventral telencephalon, resulting in an aberrant shift of their projections to more caudal cortical areas such as the somatosensory cortex (7,10).

3. Identification of other genes involved in the patterning of cortical connectivity.

The same approach has also been used to identify other genes potentially involved in the development of thalamocortical topography. Indeed, in collaboration with the groups of Drs F. Polleux (U. N.C., US) and F. Guillemot (NIMR, UK), we were able to show that the transcription factor Neurogenin-2 (NGN-2), which is expressed in the developing thalamus in a graded pattern similar to Eph receptors, is also involved in the response of thalamocortical axons to guidance cues in the early telencephalon (10). In order to characterize the potential molecular links between NGN-2 and guidance cues in the developing forebrain, we followed up on these results by looking at expression patterns of several ephrin/Eph genes (EphA3- 7, ephrin-A2,3,5, ephrin-B1-3, EphB1-4) in the thalamus and telencephalon of NGN-2 mutants. Surprisingly, we could not find any drastic variation in these patterns, suggesting that other guidance cues act downstream of NGN-2 to pattern thalamocortical projections.

We are currently trying to identify these cues by combining a gain of function approach (using Sindbis virus vectors that we have generated and that allow overexpression of NGN-2 in the embryonic brain both in vitro and in vivo) and microarray analyses.

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Publications resulting from the FMRE Research Programme in 2003:

1. Neurogenin2 Specifies the Connectivity of Thalamic Neurons by Controlling Axon Responsiveness to Intermediate Target Cues.
   Seibt J, Schuurmans C, Gradwhol G, Dehay C, Vanderhaeghen P, Guillemot F, and Polleux F.
   Neuron 39 (2003), 439-452.
2. Area-Specificity and Topography of Thalamocortical Projections Controlled by Ephrin/Eph genes.
   Dufour A, Seibt J, Passante L, Depaepe V, Ciossek T, Frisen J, Kullander K, Flanagan J, Polleux F, and Vanderhaeghen P.
   Neuron 39 (2003), 453-465.
3. Mechanisms of Patterning of Thalamocortical Projections : Intrinsic, Extrinsic, and In Between.
   Vanderhaeghen P, and Polleux F.
   Submitted.

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Shape and disparity sensitivity in monkeys: whole-brain imaging studies in awake macaques.

This year we continued to functionally characterize the far-extrastriate cortex in monkeys using fMRI. Similarly as during the first year, we emphasized upon the comparison of functional properties between the human and monkey visual system. In a first study we compared shape sensitivity in prefrontal cortex of monkeys and humans. In a second study we compared binocular disparity sensitivity in humans and monkeys. Other studies funded by GSKE are in preparation for publication.

Together with results from previous studies supported by GSKE (see list of publications from previous year and this year), the view start to emerge that during hominid evolution:

1. Functional properties of areas V1, V2, V3 and MT seem to be well preserved. Firstly, their retinotopic organization is very similar in the two species (Fize et al., 2003). Furthermore, the presence or absence of motion-sensitivity (Vanduffel et al., 2001), 3-dimensional structure-from-motion sensitivity (Vanduffel et al., 2002), and 2D-shape sensitivity (Denys et al., 2004) is very similar in these human and monkey areas.
2. Intraparietal cortex evolved much more compared to inferotemporal cortex. Indeed, on the one hand, the monkey intraparietal sulcus shows much stronger 2D-shape sensitivity (Denys et al., 2004) compared to human intraparietal areas. The opposite holds true for 3D-structure-from-motion sensitivity, which is much more pronounced in the human compared to the monkey intraparietal sulcus (Vanduffel et al., 2002). 2-D shape sensitivity, on the other hand is strong, both in monkey and human inferotemporal cortex (Denys et al., 2004).
3. Several mid-level visual areas kept some functional characteristics and lost others: An example is area V3A which has a similar retinotopic organization in the monkey and the human (Fize et al., 2003), and are both disparity-sensitive (Tsao et al., 2003). However, human V3A is sensitive for visual motion while monkey V3A is not (Vanduffel et al., 2001).

In summary, the recent developed awake monkey fMRI approach enabled us for the first time to investigating, with some confidence, possible homologies between human and non-human primate cortical areas. Indeed, exactly the same experimental paradigm and procedures can now be used to map functional characteristics in the awake monkey and human. For some areas we obtained mounting evidence that they might be homologous, others show obvious functional differences between the two species (see above and below). Obviously, many more comprehensive comparative studies are required in order to label these areas as being homologous or not. One aspect that needs to be considered in the future, is to what extent minor but inevitable differences between the functional imaging studies in the two species contribute to the observed functional differences in some brain regions. The reader should note, for example, that motivational differences might exist between human and monkey subjects. Monkeys are water-deprived in these studies and are highly motivated to obtain liquid

rewards in the MR scanner. Humans work only for small monetary rewards, which are not critical for their survival. Future work, in which even more controls will be added (e.g., such as scanning mildly fluid-deprived human subject in exactly the same way as monkeys), can add valuable insights in understanding mismatches in functional properties between corresponding human and monkey cortical areas. Evidently, genuine species differences are certainly present, given the enormous difference in brain size, and given the fact that the last common ancestor of the cercopithecoids and hominoids, and thus of macaques and humans, lived about 25 million years ago. Therefore, about 50 million years of independent evolutionary history separate humans from macaques and must have led to expanded and changed cortical territories in humans.

Shape sensitivity in monkey and human prefrontal cortex

Primate prefrontal cortex is more developed compared to that of other mammals, and it plays a key role in some of the remarkable cognitive abilities of humans. Insights regarding the evolution of cognition may emerge from comparisons of the nature of the information reaching prefrontal cortex in different primate species. Here, we investigated the responses of prefrontal cortex to images of visual objects using stimuli known to activate the ventral visual association cortex of monkeys and humans. This enabled us to compare the strength of visual signals reaching prefrontal cortex in humans and monkeys. To reach this goal, we scanned human and monkey subjects who fixated and passively viewed identical sets of visual stimuli, including familiar and novel drawings and grayscale images of objects as well as their scrambled counterparts.

In addition to the occipito-temporal and occipito-parietal activation that I described in last year's report (and see Fig. 1), monkeys' prefrontal cortex showed object-related activation, responding significantly more ($p < 0.05$ corrected for multiple comparisons) to intact than to scrambled images of objects (see Fig. 2B). The object-related activation extended from the lower bank of the principal sulcus (PS) over the inferior frontal cortex (IFC) to the anterior bank of the inferior ramus of the arcuate sulcus (irAS). The frontal activation was also observed when using different visual object and scrambled stimuli than in the original stimulus set. Under passive viewing conditions, these object-related prefrontal regions behaved consistently. The response was greater to intact images than to scrambled ones and more to grayscale images than to drawings. The interaction between these two factors was stronger in the principal sulcus than in the arcuate sulcus.

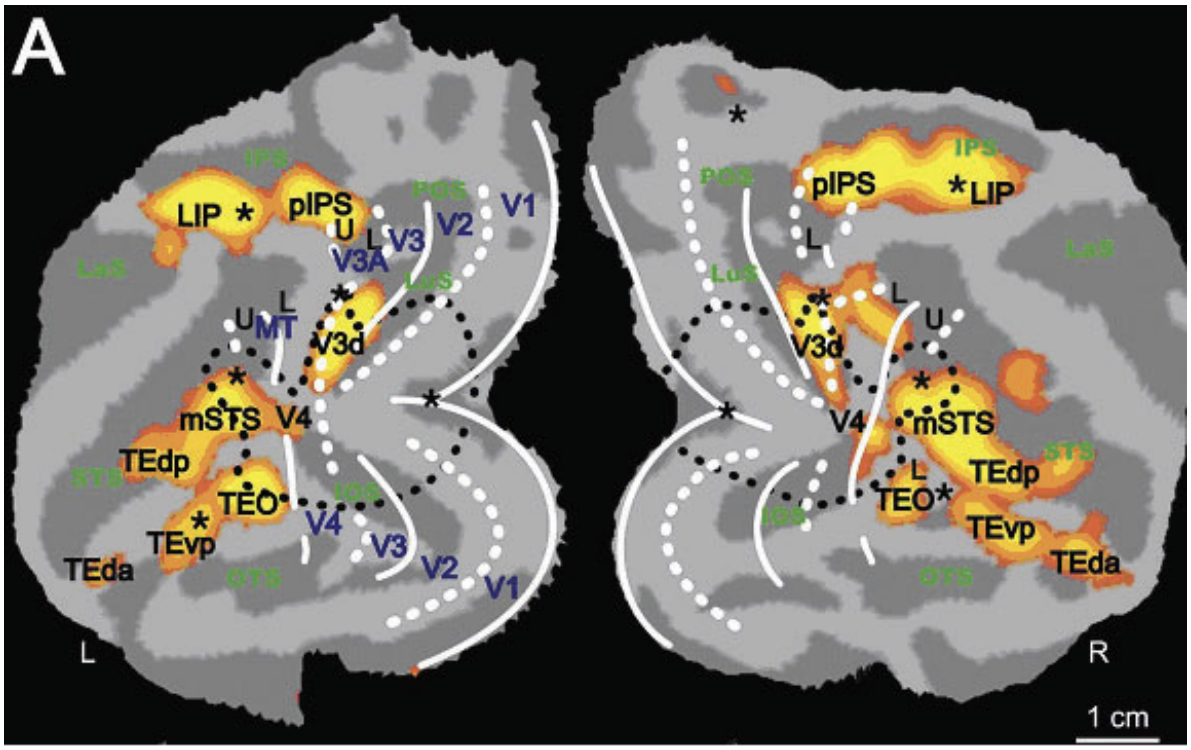

Fig. 1. Shape sensitivity in occipital cortex of the monkey (group analysis, n=4, comparing images of objects with scrambled images of the same objects. Notice the surprisingly strong intraparietal shape-sensitivity.

In humans a much more restricted prefrontal object-related activation was observed (see Fig. 2A). This contrasts with the monkeys in which many prefrontal voxels showed the effect of scrambling. In order to estimate the extent of cortical activation, we determined the total volume showing significant activation and expressed this as a percentage of the overall volume in prefrontal cortex. For the macaque, the prefrontal activation for the two hemispheres combined (447 mm3) was 8% of the total volume of prefrontal cortex (5310 mm3). For the human, the prefrontal activation was only 0.9% of prefrontal cortical volume (1593 mm3 activation/177000 mm3), which is an order of magnitude smaller than that of the macaque.

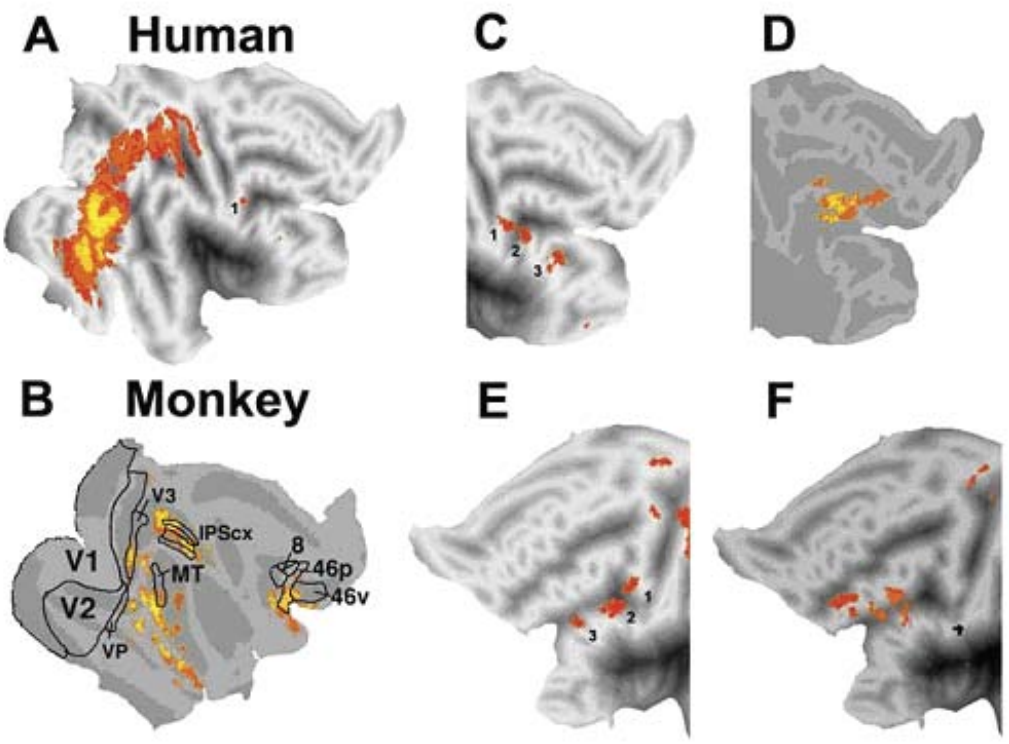

Fig. 2. Fig. 4. Flatmaps comparing human and monkey prefrontal activation. (A) SPM of the main effect of scrambling in human (group, fixed effect, p < 0.05 corrected for multiple comparisons) on flattened right hemisphere of the human Colin atlas map (Caret software). (B) SPM of the main effect of scrambling in monkey (group, fixed effect, p < 0.05 corrected for multiple comparisons) on flattened right hemisphere of the Macaque.F99UA1 atlas map. (C) Activation in right prefrontal cortex of humans: (group, fixed effect, main effect of scrambling, p < 0.001 uncorrected). (D) Predicted activation of human prefrontal cortex obtained by warping monkey object-related activation for right hemisphere. 1: posterior MFG sites (BA 44/9), 2: IFG sites in BA45/47.

Lowering the threshold to $p < 0.001$ uncorrected for multiple comparisons did not reveal any additional prefrontal sites (Fig. 2C; small patches on the lower right of the map are in orbito-frontal cortex and cannot be considered significant in absence of a priori information).

Thus the prefrontal activation by object images in humans is small, both in extent and in magnitude. This difference in visually-driven activation between humans and monkeys is unlikely to depend on the novelty of the stimuli nor on the experience with the scanning environment.

To control for possible differences in attention during passive viewing between the two species, we compared the effect of scrambling in the two species while monkeys and humans performed a demanding task with a small central stimulus. In monkeys, the object-related activation remained significant. Yet, the task itself produced a stronger activation of the principal sulcus site. In humans attention had no significant effect on the small object-related activation observed in left IFG. Thus the difference in visual activation of prefrontal cortex cannot be attributed to differences in attention.

Our results in the monkey are in excellent agreement with many anatomical showing direct connections from V4 and the infero-temporal complex to the prefrontal cortex below the PS and in front of the inferior ramus of the AS. Consistent with this input from areas with a high

proportion of shape-selective neurons, many physiological studies have reported a high incidence of responses to complex visual shapes in macaque IFC. Scalaidhe estimated the incidence of object-selective neurons to be smaller in IFC than in IT. This is in accord with the relative magnitude of object-related activation in infero-temporal versus prefrontal cortex of the monkey observed in the present study.

The small magnitude of object-related human prefrontal activation in the present study is consistent with the results from other human imaging studies which used similar paradigms, included prefrontal cortex in their analysis, and failed to observe a prefrontal activation by shape stimuli.

Several explanations, not mutually exclusive, can be advanced for the species difference in prefrontal function we observed. First the type of information reaching prefrontal cortex may be different, being more polysensory than visual in humans. This view is supported by our observation that object-related activation in human occipito-temporal cortex (LO complex) does not extend nearly as far anteriorly in humans (terminating about 4 cm posterior to the temporal pole). Thus, assuming that the complete extent of human and monkey infero-temporal cortex projects equally to prefrontal cortex, there will be relatively less visual input in human compared to monkey prefrontal cortex. Selective gating of the visual information reaching prefrontal cortex is an alternative. By this hypothesis, monkey prefrontal cortex would process visual information more automatically than human prefrontal cortex. At present we can only speculate about the nature and the origin of such gating signals. Finally, prefrontal cortex is proportionally larger in humans than in macaques suggesting that new prefrontal areas or subareas may have emerged in humans. Thus the object-related prefrontal activation might be equally large in terms of prefrontal cortical regions shared by humans and monkeys, but appear smaller in humans because of the new areas/subareas that emerged in humans.

Our finding that visual object-related activation is much stronger in monkey than in human prefrontal cortex is an important element for understanding the evolution of cognition. It complements the demonstration that similar prefrontal regions in the two species are engaged in set shifting (Nakahara et al., 2002). Both studies show the strength of the comparative functional imaging approach, as the evolution of prefrontal cortex function can be assessed by new powerful tools in addition to cytoarchitectonics and anatomical size comparisons.

In a second study, we investigated binocular disparity in monkeys and humans

Our perception of shapes and surfaces in 3D space is the intuitive basis for our understanding of the physical world. The surface structure of an object provides a powerful identification tool and also indicates how an object should be grasped and handled. A powerful cue to 3D structure is binocular disparity or the difference between the images in the two eyes. Disparity specifies not only the depth of each point in the visual array, but also higher-order surface properties such as edges, surface orientation, and shape. Cells sensitive to disparity edges have been reported in areas V2, MT, and MSTl. Tuning to disparity-defined 3D surface orientation and 3D curvature have been found in the caudal intraparietal sulcus (CIPS) and area TEs.

The relative contribution of different areas to coding different higher-order surface properties-the global architecture of stereopsis-remains unclear. Single-unit data from monkeys suggest that disparity processing is widely distributed throughout the visual cortex. In contrast to these data, several fMRI studies of human visual cortex have found that the BOLD signals elicited during stereopsis is localized to area V3A and cortex adjacent to the intraparietal sulcus. Human MT+ was not prominently activated in any of these studies. Some groups have reported additional activation in the lateral occipital complex, in response to random-dot stereograms of complex objects. It is difficult to directly compare fMRI studies in humans with electrophysiological studies in macaques, because in such comparisons species differences are confounded with technique differences. In this study, we therefore examined fMRI activation to stereoscopic stimuli in the visual cortex of the alert macaque monkey and compared it to that in the human.

In macaques, we found strongest activation to near/far compared to zero disparity in areas V3, V3A, and CIPS. In humans, we found strongest activation to the same stimuli in areas V3A, V7, the topological 'equivalent of V4d, and a caudal parietal disparity region. Thus, in both primate species a small cluster of areas at the parieto-occipital junction appears to be specialized for stereopsis.

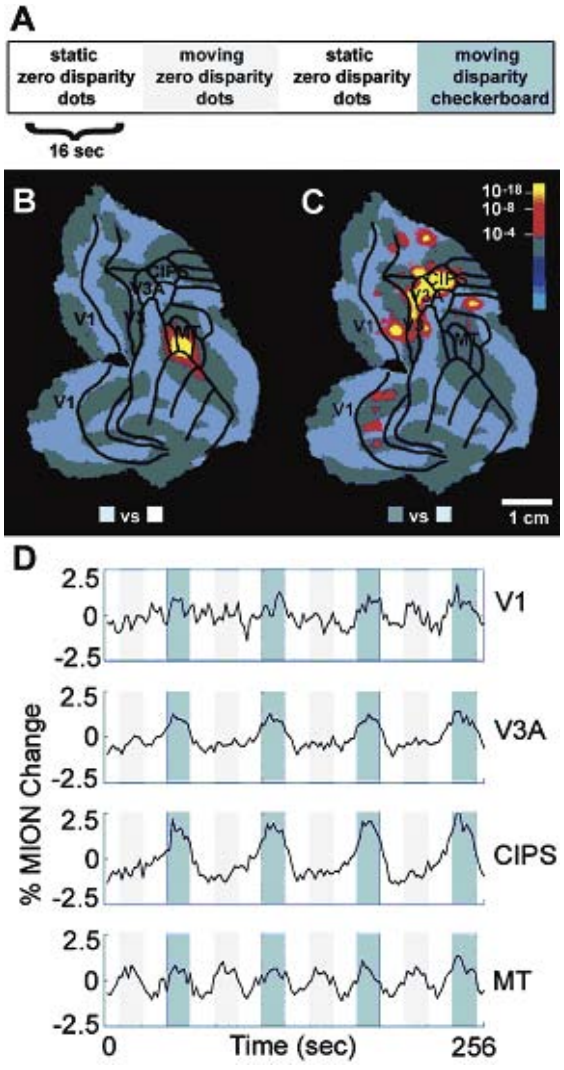

Fig. 3 The response to a coherently moving disparity pattern in monkey MT and V3A/CIPS. Coherent global motion was visible in the monocular carrier. A: diagram of the stimulus conditions. B statistical parametric maps: moving zero disparity versus static zero disparity. C: moving disparity checkerboards versus moving zero disparity. D: timecourses

Our observations suggest that the posterior parietal lobe is crucial to cortical 3D processing using disparity as cue. These results raise at least three questions. (1) What is the functional correlate of the disparity fMRI signal (e.g., absolute versus relative disparity, attention, eye movements, etc.)? (2) How does the pattern of disparity-based fMRI activity in monkeys compare to results from single units? (3) How does the architecture of disparity processing in monkeys compare to that in humans?

What Is the Source of the Disparity-Related fMRI Signal? There are at least four possibilities. (1) Increased fMRI activity to near/far compared to zero disparity could reflect the concentration of near and far disparity-tuned cells in a region. (2) The activity could reflect the processing of relative disparity signals and/or high-level shape extraction. (3) The activity could be due to secondary planning and execution of eye movements elicited by the disparity stimulus. (4) The activity could be caused by a general increase in attention during the near/far disparity condition compared to the zero disparity condition.

The last two possibilities appear unlikely. Monitoring of eye movements inside the scanner indicated no difference in the magnitude of horizontal or vertical eye movements during near/far compared to the zero disparity conditions. Furthermore, explicit imaging of activity produced by vergence eye movements showed that vergence eye movements and stereoscopic surfaces activated largely nonoverlapping regions of cortex. However, we cannot rule out the possibility that eye movement differences between the disparity-rich and zero disparity conditions contributed to some of the activation patterns we observed.

It is also unlikely that apparent disparity sensitivity was due solely to increased attention (possibility 4 above). In the human, disparity-driven activation was weaker when attention was diverted by a demanding foveal task. Nevertheless, in both the monkey and the human, the overall topographic pattern of activity produced when the subject performed a difficult bar-orientation discrimination task during disparity scanning was similar to that obtained when the subject performed a passive fixation task.

This leaves the first two possibilities: near/far cells and/or cells sensitive to relative disparity produced the disparity-driven fMRI activity. The results of the relative disparity experiment indicate that disparity activation in macaque areas V3, V3A, and CIPS and human areas V3A, V7, and V4d-topo was most likely due to a combination of both absolute and relative disparity processing (possibilities 1 and 2), while disparity activation in macaque area MT and in human areas MT+ was due to absolute disparity processing. Furthermore, relative disparity activity in macaque areas V3, V3A, and CIPS was not due to general scene segmentation processes, but was due to 3D scene segmentation specifically, since we found no activation in these areas to an orientation-defined checkerboard versus a uniform-orientation pattern.

Humans and macaques have evolved independently of each other for over thirty million years. Thus, it is unlikely that there exists a one-to-one homology between all cortical areas in the two species. The current results corroborate this view and underscore the importance of doing fMRI in monkeys rather than in humans, if one's goal is to obtain an activity map to guide single-unit studies. In both species, V3A was activated by disparity. But the distribution of disparity

activity was different in the two species: the strongest disparity activation occurred in area CIPS in macaques and in the V4d topolog in humans.

The disparity sensitivity in area V3A, common to both humans and monkeys, is interesting from an evolutionary perspective. Although human and macaque V3A are topographically homologous and have a similar retinotopy (both contain a contiguous representation of the entire contralateral visual field), an important functional difference exists between them: human V3A is moderately motion sensitive. The finding here that both human and macaque V3A are disparity selective suggests that stereopsis may be a more evolutionarily fundamental function of area V3A, compared to motion processing.

The activation patterns to stereoscopic stimuli that we have observed in the macaque brain strongly emphasize the importance of areas V3, V3A, and CIPS in 3D processing. They provide single-unit physiologists with a new roadmap, and detailed physiological study of these areas may reveal the circuits by which single cells and groups of cells generate the percept of surfaces in space.

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

It is well established that other cues besides binocular disparity can signal depth structure, e.g. motion, texture, shading and other figural depth cues, and thus the question arises whether IT neurons also code depth from these other cues. So far, we have determined whether IT neurons also code for 3D shape-from-texture. In a first series of experiments, described in the 2002 annual report, we compared the responses to texture patterns that produce a percept of a convex or a concave surface, i.e. a single curved surface. In that study, effects of the texture cue at the single cell level were, although reliable, weak, agreeing with the results of a monkey psychophysical study that also showed a significant, albeit weak, processing of the texture depth cue.

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 (r = .80), 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 IT there is convergence of different depth cues at the single cell level. We have 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 IT 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. Interestingly the tilt selectivity was significantly greater for monocular compared to the binocular presentations. This is expected when these neurons code for 3D tilt and not merely texture variations, since the 3D percept is more salient for monocular than for the binocular presentations since in the latter case there is a conflict with the disparity cue signaling a flat surface. These results convincingly demonstrate that a ventral visual area, i.e. IT, code for 3D texture cues.

Current work is addressing the coding of another monocular depth cue: shading.

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 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.

3. Perceptual learning 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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