Geneeskundige Stichting Koningin Elisabeth

Fondation Médicale Reine Elisabeth

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Geneeskundige Stichting Koningin Elisabeth

2000

Inleiding

Verslag Activiteiten van de GSKE – FMRE

In het jaar 2000, werden de sinds 1999 geprogrammeerde onderzoeksactiviteiten verdergezet. Het wetenschappelijk rapport van de verschillende ploegen, geeft een stand van zaken over de evolutie van hun werk tijdens de driejaarlijkse periode 1999-2001.

Twee universitaire ploegen (Prof. Dr. C. Erneux - ULB en Prof. Dr. E. De Schutter - UIA) hebben, gezien de uitstekende kwaliteit van hun onderzoek, een éénmalig krediet ontvangen van het resterende kapitaal van Polak-Bosquet.

De volledige lijst van de begunstigden is de volgende:

C. Ampe (RUG);
W. De Potter (UIA, Antwerpen);
E. De Schutter (UIA, Antwerpen);
G. Ebinger (VUB, Brussel);
C. Erneux (ULB, Bruxelles);
E. Godaux (U.M-H, Mons);
C. Godfraind (UCL, Bruxelles);
A. Goffinet (FUNDP, Namur);
J.M. Kaufman (RUG/UZ Gent);
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F. Vandesande (KUL, Leuven);
R. Vogels (KUL, Leuven).

Fondation Médicale Reine Elisabeth

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Introduction

Rapport d'Activités de la FMRE - GSKE

L'année 2000 a connu la poursuite des activités de recherches programmées depuis 1999. Le rapport scientifique des différentes équipes fait état de l'évolution de leurs travaux au cours de la période triennale 1999-2001.

Deux groupes universitaires (Prof. Dr. C. Erneux - ULB et Prof. Dr. E. De Schutter - UIA) ont bénéficié, vu l'excellence de leur activité, d'un crédit d'encouragement unique, prélevé sur le reliquat du capital Polak-Bosquet.

La liste complète des bénéficiaires est la suivante:

C. Ampe (RUG);
W. De Potter (UIA, Antwerpen);
E. De Schutter (UIA, Antwerpen);
G. Ebinger (VUB, Brussel);
C. Erneux (ULB, Bruxelles);
E. Godaux (U.M-H, Mons);
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P. Van Hecke (KUL, Leuven);
F. Vandesande (KUL, Leuven);
R. Vogels (KUL, Leuven).

De talrijke en kwalitatief zeer hoge publicaties, die hernomen zijn aan het einde van elk rapport, telkens met de vermelding van de GSKE, getuigen van de vitaliteit en de creativiteit van de ploegen die geselecteerd werden door het Wetenschappelijk Comite.

Wij stellen vast dat de GSKE de ontwikkeling in ons land van een sterk wetenschappelijk netwerk in het domein van de neurowetenschappen heeft bevorderd. Deze vaststelling stimuleert het Wetenschappelijk Comite om dit thema te behouden in de komende jaren.

Inderdaad, de GSKE is de enige Belgische Instelling, die uitsluitend onderzoeksprogramma's in de neurowetenschappen ondersteunt. De stichting verwerft hierdoor een originaliteit en een cohesie die de eindresultaten alsmaar doen verbeteren.

De onderzoeksthema's, fundamenteel gericht naar de grote hedendaagse problemen, worden aangepakt door toepassing van meer adequate middelen opdat onderzoekers hun project zo goed mogelijk zouden kunnen tot een goed einde brengen.

Wij houden eraan, in naam van alle verschillende ploegen, onze erkentelijkheid te betuigen aan de leden van de Raad van Beheer voor hun doorzicht, hun begrip en hun edelmoedigheid.

Prof. Dr. Th. de Barsy

Brussel, 28 februari 2001

L'abondance et la qualité des publications reprises à la fin de chaque rapport, mentionnant chaque fois le soutien de la F.M.R.E., témoignent de la vitalité et de la créativité des équipes sélectionnées par le Comité Scientifique.

On constate que la F.M.R.E. a favorisé le développement dans notre pays d'un réseau scientifique solide dans le domaine des neurosciences. Cette observation encourage le Comité Scientifique à maintenir ce thème dans les années à venir.

La F.M.R.E., en effet, est la seule Institution Belge à soutenir exclusivement des programmes de recherche en neurosciences. Elle acquiert de ce fait une originalité et une cohésion dont les fruits vont croissant.

Les thèmes de recherche fondamentale orientés vers les grands problèmes de notre époque sont abordés avec des moyens plus adéquats pour permettre aux chercheurs de finaliser au mieux leur projet.

Nous tenons à exprimer, au nom des différentes équipes, notre reconnaissance chaleureuse aux membres du Conseil d'Administration pour leur clairvoyance, leur compréhension et leur générosité.

Prof. Dr. Th. de Barsy

Bruxelles, 28 février 2001

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Report of the Research Group of

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Collaborations with:

Prof. Dr. R. Fässler *(Lund University, Sweden)*
Prof. Dr. F. Gertler *(MIT, Cambridge, USA)*

The role of profilins and EVL during neurite extension and filopodial growth cone formation (GKSE 1999-2001)

Background

In this project we address the roles of profilins and their partner protein EVL in actin based cell motility during neurite extension. We also investigate the regulation of these interactions by signal transduction events. Profilins are actin binding proteins of which three isoforms have now been identified in vertebrates profilin I, Ia and IIb (Lambrechts et al., 2000a). They are capable of binding a variety of regulatory ligands and are therefore ideal players to act at crossroads of signal transduction pathways influencing actin dynamics and modulating cell motility (Sohn and Goldschmidt-Clermont 1994). These ligands include phosphoinositides and proline-rich proteins (Lambrechts et al., 1997) of which Ena/VASP proteins form a group of interest for this project. The members of this group: Mammalian enabled (Mena), Vasodilator stimulated phosphoprotein (VASP) and Ena/VASP like protein (EVL) (Gertler et al., 1996) show an overlapping tissue specific expression pattern but the relative abundance in tissues is different (Lanier et al., 1999). They share a typical three-domain structure. The N-terminal domain interacts with focal adhesions proteins (zyxin, vinculin) (Reinhard et al., 1996) or receptor associated proteins (such as Fyb/slap, Krause et al., 2000). Very recently it was shown that EVL interacts, via this EVH1 domain, specifically with the transmembrane form of semaphorin 6a (Klosterman et al., 2000) that acts as a repulsive axon guidance cue. The middle domain is proline-rich and binds SH3-domains and profilins in a competitive manner (Lambrechts et al., 2000b), the C-terminal domain mediates oligomerization (Getrler et al., 1996) and is also involved in direct modulation of actin polymerisation (Bachman et al., 1999, Lambrechts et al., 2000b, Harbeck et al., 2000). In addition these proteins are substrates for cAMP-dependent kinase (PKA) (Hallbrügge et al., 1996, Gertler et al., 1996, Lambrechts et al., 2000b).

Mena has been implicated in neural development. Mena-/- mice are viable but their axons fail to cross the midline. Crosses with profilin I -/+ result in embryonic lethality. These mice lack neural tube closure (Lanier et al., 1999) a process known to be dependent on actin polymerization. At the subcellular level Mena is enriched at the tips of growth cone filopodia in embryonic hippocampal neurons. The role of EVL in neurite extension is unclear but the protein is strongly expressed in neurons of adult rat brains (Ohta et al., 1997).

Profilin IIa is a neural specific form and a preferred partner for EVL

In the first part of this project we examined the expression patterns of profilins during brain development and characterised the differences in biochemical properties of profilins. Whereas profilin I is expressed ubiquitously profilin IIa is the neuronal form. These results have been published recently (Lambrechts et al., 2000a). Also EVL expression in brain is developmentally regulated (Lanier et al., 1999) and the time course of its expression nearly coincides with profilin IIa (Lambrechts et al., 2000a). We biochemically characterised EVL which is also a substrate for protein kinase A. It preferentially associates with profilin IIa and the interaction is independent of the phosphorylation state. Interestingly EVL also associates with specific SH3-

domains, most notably that of N-src (but not of src) the neuronal splice variant of src. This interaction is inhibited upon phosphorylation of EVL and SH3-domains compete with profilin for binding to EVL. This protein translocates to the tip of microspikes upon stimulation of cells (Lambrechts et al., 2000b).

The effects of profilin and EVL on actin dynamics

During the course of our experiments we discovered that EVL nucleates actin polymerisation under physiological conditions and that phosphorylation of EVL by PKA reduces this capability (Lambrechts *et al.*, 2000b). Since profilin is also an actin binding protein it is important to investigate the effect of profilin IIa on EVL-mediated actin polymerisation. This research is still in progress but preliminary results indicate complex kinetics, changing in function of profilin concentration.

A differential role for profilins during neurite outgrowth and axon in PC12 cells?

Given the role of profilin I in neuronal development (Lanier *et al.* 1999) and the expression patterns of EVL and profilin IIa in the developing brain (see above), it is important to address the differential roles of the profilins in neuronal cells. We chose the pheochromocytoma PC12 cell line as a model system. This cell line extends neurites upon stimulation with nerve growth factor (NGF). Following stimulation profilin IIa expression is induced (unpublished results) whereas profilin I is present both in the undifferentiated and differentiated cells. The three mammalian ena/VASP members are expressed (Goldberg *et al.*, 2000, Otha *et al.*, 1997). Interestingly, a recent study showed that the subcellular location of Mena but not of VASP changed upon stimulation of these cells with NGF, the location of EVL, however, was not documented in this study (Goldberg *et al.*, 2000).

We devoted part of time last year to develop tools amenable for cell biological experiments. For many years a major problem in studying subcellular localisation of profilins has been the lack of antiprofilin antibodies suitable for immunofluorescence staining of cells. In addition tagged forms (or fusions with green fluorescent proteins, GFP) of profilins have altered biochemical properties (e.g. the poly-L-proline binding capability is reduced by a factor of approximately 100, Jonckheere *et al.*, unpublished). Thus one has to question their application by transfection experiments in cells. Therefore we now focus on vectors having IRES sequences (internal ribosome entry sites) between the profilin and GFP coding sequences enabling us to select cell populations with varying levels of profilin I or IIa expression (this approach still does not allow to observe subcellular localisation of profilins). The wild type isoforms have already been brought into the system. We now also possess profilin I mutants with reduced affinity for actin and/or phosphatidylinositol-4,5-bisphosphate (Lambrechts *et al.*, submitted) and a profilin IIa mutant with strongly reduced affinity for proline-rich ligands (Jonckheere *et al.*, unpublished). These will be cloned in the IRES system and transfected in the PC12 cells. The behaviour of these cells upon stimulation of NGF will be observed and correlated with the expression level of the particular isoform (or mutant) and with the localisation of ena/VASP members and EVL in particular. In addition, parallel to this study we will perform a proteomics analysis of profilin I and

IIa affinity purified fractions of (non-transfected) PC12 cells in function of time after NGF stimulation enabling to identify their cellular partners.

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Papers from the research group realised within the programme

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- Lambrechts, A., Jonckheere, V., Dewitte, D., Vandekerckhove, J. and Ampe, C. Mutational analysis of human profilin I identifies Arg88 and Arg136 for PI(4,5)-P2 binding. Submitted.

Report of the Research Group of

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Part 1: Characterisation of noradrenergic large dense-cored vesicles

It is generally accepted that neurons can secrete a cocktail of peptide and non-peptide or classical neurotransmitters from at least two types of organelles, i.e. the small synaptic vesicles (SSV) and the large dense-cored vesicles (LDV). According to the current views, the classical transmitters, such as acetylcholine (ACh), noradrenaline (NA), glutamate and GABA, are stored in and released from the SSV. On the other hand, the neuropeptides are stored in and released from the LDV. Exocytosis from SSV and LDV is considered to be differentially regulated and to take place at different release sites. However, there are indications to believe that this general concept of neurotransmitter release is oversimplified and that different neural systems with different physiological functions in the organism have developed variations on this commonly accepted model. This is certainly the case for the peripheral noradrenergic neurons, the subject of our research. Noradrenergic neurons store their classical transmitter NA not only in the small dense-cored vesicles (SDV), but, together with the neuropeptides, also in LDV. In addition, in the previous years we were able to demonstrate that in noradrenergic neurons NA is exclusively released from the LDV. Despite their numerical majority, the SDV are not directly involved in noradrenergic neurotransmission. Now we want to come to an identification of all proteins present on the LDV-membrane in order to obtain a detailed view of the intraneuronal dynamics of this vesicle-population, which seems to be of crucial importance for the noradrenergic neurotransmission.

In a first approach LDV were purified using sucrose/D_2O gradient centrifugation. This method is actually considered as being the most efficient procedure to purify LDV. This purified LDV fraction was analysed with 2D-gelelectrophosesis. Via Western-Blot with antibodies to well known membrane-bound vesicle-proteins we were able to assign the identity of several spots. In order to obtain the identity of the unknown spots, we subjected them to amino-acid analysis. Unfortunately, it turned out that most of these spots were due to mitochondrial contamination. We had to conclude that the current purification procedure for LDV was insufficient to obtain our goals and that a more specific procedure had to be developed.

A new purification procedure for LDV based on immuno-affinity chromatography was set up. For this purpose polyclonal antibodies to synaptobrevin, a protein known to be present on LDV-membranes, were prepared and coupled to CNBr-activated Sepharose beads. These beads were then packed in a column on which a post-mitochondrial supernatant obtained from bovine splenic nerves was loaded. However, the amount of purified LDV obtained by this approach was too limited to perform an elaborate analysis. An alternative procedure in which we labeled the LDV with magnetic beads, was more succesful. In this procedure a post-mitochondrial supernatant of bovine splenic nerves was incubated with polyclonal antibodies to synaptobrevin and the monoamine transporter VMAT2 (also prepared in house), followed by an incubation with magnetic beads on which anti-rabbit IgG's were coupled. The magnetically labeled LDV were then separated from the contaminating organelles in an magnetic field. In order to estimate the degree of purity of the LDV-fraction obtained by this method, the enrichment of different LDV-membrane markers has to be calculated. Therefore, ELISAs to dopamine-beta-hydro-

xylase and VMAT2 are developped. The final experiments are now being performed. The analysis of the purified LDV fraction via 2D-gelelectrophoresis will be performed as soon as we will have an objective idea of the degree of purity of this LDV-fraction. An advantage of this procedure is that this LDV-fraction can also be analysed by electron microcopy. Electron microscopical images point to an almost exclusive presence of LDV in this fraction.

In the mean time, we could assign three new proteins to LDV-membranes.

- As was already described in our previous report, we were able to demonstrate the presence of N-type voltage operated calcium channels (N-type VOCC) on LDV-membranes. In order to obtain additional evidence for this finding we demonstrated the presence of N-type VOCC in the LDV-fraction, purified by the magnetic bead method described above. In addition, in collaboration with Prof. J. Quatacker (RUG) we could confirm the presence of N-type VOCC on LDV-membranes by electron microscopy (Partoens et al., submitted).

- Calcium-dependent activator protein for secretion (CAPS) is a neuro-endocrine cell- specific protein that functions at a calcium dependent triggering-step before dense-cored vesicle exocytosis. It is demonstrated that CAPS binds selectively to the plasma membrane and to LDV but not to SSVs. It is hypothesised that CAPS is involved in modulating the curvature of the LDV-membrane to optimise lipid-bilayer interaction between the vesicle and plasma membrane before exocytosis. In a collaboration with Prof. Dr. Martin (University of Wisconsin, US), who provided us with CAPS-specific antibodies, we tried to find evidence for the presence of CAPS on LDV, and its absence from SDV in peripheral noradrenargic neurons. Analysis of a microsomal fraction obtained from rat vas deferens after sucrose gradient centrifugation revealed that CAPS was enriched in the LDV-fraction, and depleted from the SDV-fraction. We also found CAPS enriched in the highly purified LDV-fraction from bovine splenic nerve. These data are in line with the current view. Electron microscopical confirmation should follow these biochemical data.

- The DFNA5 gene is one of the many genes responsible for non-syndromic hearing loss. This gene was identified by the Lab. of Medical Genetics (UIA). Up to now and in spite of extensive computational analysis no putative physiological function could be assigned to DFNA5. In a collaboration with Dr. L. Van Laer (Lab. of Medical Genetics, UIA), who provided us with antibodies to DFNA5, we are now trying to elucidate the subcellular localization of DFNA5 in bovine adrenal medulla. Our preliminary data point to the presence of DFNA5 on LDV-membranes.

Part 2 : Chromogranins as neuro-immunomodulators

Chromogranins are considered to be precursor molecules for biologically active peptides. Their processing occurs at multibasic sites by endogenous proteases of the kex2 family. The chromogranins are present in the large dense cored vesicles of many endocrine cells and neuronal tissues, where they are co-stored and co-released with amine neurotransmitters, neuropeptides and peptide hormones. They are also considered as a new class of putative immunomodulatory molecules. Some of the chromogranin-derived peptides have already been shown to possess immunomodulatory properties. Pancreastatin ($pCgA_{240\text{-}289}$) stimulates the T-cell proliferation (Haberstock-Debic et al., 1997), WE-14 ($hCgA_{324\text{-}337}$) modulates the histamine release from mast cells (Forsythe et al., 1997) and secretoneurin ($SgII_{154\text{-}186}$) is a chemoattractant for monocytes (Reinisch et al., 1993).

During the last year, 4 new peptides derived from chromogranin B (CgB) and one derived from chromogranin A (CgA) were isolated.
Z. Wang et al. (in press) reported the isolation of 3 new peptides from the porcine adrenal medulla, located at the C-terminal part of CgB. These peptides were obtained by size-exclusion chromatography, immuno-affinity chromatography and reversed phase chromatography and identified by electrospray tandem mass spectrometry and Edman degradation. One peptide was identified as SR-17 ($pCgB_{586\text{-}602}$) and is phosphorylated at one or two serine residues. Another one, HQ-34 ($pCgB_{603\text{-}636}$) with a molecular mass of 3815.56 is oxidized at methionine. A third one, KR-11 ($pCgB_{637\text{-}647}$), is a secretolytin-like peptide that is 2 amino acids shorter than the bovine secretolytin ($bCgB_{614\text{-}626}$). This is the first report giving evidence of the fact that the C-terminal part of CgB, the homologue of human CCB ($hCgB_{597\text{-}653}$), is further proteolytically processed to 3 smaller peptide fragments.

MK-27 ($pCgB_{1\text{-}27}$) is a new peptide from the N-terminal part of porcine CgB. This is the first N-terminal CgB-peptide that is shown to be produced *in vivo*. Using ELISA against the peptides MK-27, SR-17 and KR-11, we showed that CgB is processed to its smaller peptides during its axonal transport, using the porcine splenic nerve as a model. We also demonstrated the release of the 3 peptides from the porcine splenic nerve, using the pig spleen perfusion technique with frequency-dependent stimulation of the nerve and in the presence or absence of the α-blocking agent phentolamine (Z. Wang et al., in preparation; Depreitere et al., in preparation a+b).

MK-27 and SR-17 have been shown to be good substrates for the enzyme dipeptidyl peptidase IV (DPP IV), also known as CD26, a lymphocyte surface glycoprotein. This is a membrane-associated serine-protease which cleaves off dipeptides from the N-terminus of peptides and it has a high selectivity for proline and a lower selectivity for alanine in the penultimate position (De Meester et al., 1999). Using this enzyme, MK-27 is processed to VK-25 by cutting off the dipeptide MP- and SR-17 is processed to ER-15, losing the dipeptide SA-. The corresponding k_{cat}/K_m values are $9*10^4$ $M^{-1}s^{-1}$ for MK-27 and $1*10^4$ $M^{-1}s^{-1}$ for SR-17. This specificity constant reflects the efficiency to hydrolyse the peptides at physiological concentrations. MK-27 and SR-17 processing rates are 10-100 times slower than the best reported peptide substrates,

including NPY. After DPP IV/CD26 activity, NPY becomes an angiogenic factor (Zukowska-Goric et al., 1998). Likewise it is hypothetised that the DPP IV processing of the peptides MK-27 and SR-17 may be part of a physiological mechanism that modulates their biological activity as potential immunomodulators (Depreitere et al., in preparation a+b).

Immunohistochemical analysis of porcine spleen sections revealed the presence of both the MK-27 and SR-17 sequence / peptide exclusively in nerve terminals localised in the reticuloendothelial compartment lining the Billroth cords, in the trabeculae, and in the vessel wall of central arteries. Immunoreactivity was also seen in the larger branches of the splenic nerve. In contrast, pancreastatin (a chromogranin A fragment) imunostaining could not be detected in nerves or nerve endings but was localised in specific subsets of leukocytes in the white pulp and red pulp. This interesting observation was confirmed by in-situ hybridisation (in collaboration with Dr. L. Arckens & Prof. Dr. F. Vandesande, K.U.L.), implying that the observed pancreastatin immunoreactivity in these bleukocytes was indeed produced by these cells and was not the result of an uptake process of chromogranin originating from other sources.

GN-17 ($pCgA_{292-308}$) is a new peptide derived from CgA, located in the middle part of the molecule. It shares a similar concensus motif with many substrates of protein kinase II. Preliminary results confirm that this peptide inhibits in a dose-dependently way the casein kinase II (CKII) activity, whereas peptide WE-14, used as a negative control, has no effect. The mechanism of inhibition remains to be further investigated. The interesting part is that this CKII-like activity has been found on the cell surface of both T-lymphocytes and mast cells and that phosphorylation sites of CKII are present on the T-cell receptor. This may be suggestive for a modulating action of GN-17 on T-lymphocyte function.

We also compared the antibacterial activity against Micrococcus luteus of the lysate of porcine and bovine chromaffin granules. In the bovine adrenal medulla, antibacterial activity was found; this is in accordance with the known antibacterial activity of bovine secretolytin. No activity against Micrococcus luteus was found in porcine chromaffin granule extracts, nor in preparations of the pure synthetic porcine secretolytin-like peptide KR-11. We therefore conclude that the secretolytin-like peptide KR-11 does not possess antibacterial activity. This activity is probably situated in the C-terminal part of secretolytin, where KR-11 differs in one amino acid from the real (=bovine) secretolytin.

In conclusion : Several new peptides derived from CgB and one from CgA have been identified in porcine chromaffin granules and some functional properties could be estabilised. Two of them are good substrates for DPP IV/CD26 and another one is an inhibitor of the CKII activity. Three of them are released from the splenic nerve after electrical stimulation.

Publications 2000 :

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Report of the Research Group of

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INTRODUCTION

The alignment of parallel fibers along the length axis of a folium and perpendicular to the plane of Purkinje cell dendrites is the basis for most theories about cerebellar functioning (Marr 1969; Eccles 1973; Braitenberg 1983; Braitenberg et al. 1997). Nevertheless, recent experimental studies have raised doubts about the effectiveness of parallel fibers in activating Purkinje cells (Bower and Woolston 1983; Cohen and Yarom 1998). As these results could be caused by several different mechanisms, including for example failure of transmission along parallel fibers, it is important to establish what parallel fiber activity patterns in response to natural stimuli look like. In our studies we use Golgi cells as sensors for such activity and use a combination of experimental and modeling studies to investigate their response patterns to both punctate and semi-continuous stimuli. Here we only report on our recent experimental findings.

This work is an extension of previous studies by our group on Golgi cell dynamics. These originated with a modeling study (Maex and De Schutter 1998) which suggested that parallel fibers synchronize the spontaneous activity of Golgi cells. This prediction was confirmed experimentally in the anaesthetized rat (Vos et al. 1999a; Maex et al. 2000). Additional experimental studies of the response to punctate tactile stimulation led to the suggestion that parallel fiber activation of Golgi cells was responsible for their large receptive fields (Vos et al. 1999b). Moreover, the latter study suggested that a particular response pattern, consisting of two early double peaks, was caused by direct mossy fiber activation of the Golgi cell while the other response patterns were presumably due to a mixture of mossy fiber and parallel fiber activations (Vos et al. 1999b; Vos et al. 2000). One of the goals of the present study is to exactly quantify the respective contributions of these two pathways.

Another prediction of the network model was that the synchronization of Golgi cells would increase with higher levels of mossy fiber activation of the granular layer. While we found indirect evidence for this in our previous experimental studies (the synchrony was positively correlated to Golgi cell firing rate; Vos et al. 1999a), we would like to demonstrate this relation more directly by activating the circuit. Unfortunately one cannot do this using punctate stimuli, because strong phase locking of the responses to the stimulus makes it impossible to analyze any contributions from granular layer network dynamics (results not shown). As shown in the results section, a sweeping stimulation by a paintbrush provided for a semi-continuous activation of mossy fibers with the desired properties. Moreover, the effects of this stimulus on Golgi cell synchronization can only be explained by taking into account the differences in the balance between mossy fiber and parallel fiber input activating each Golgi cell.

Overall our results demonstrate that parallel fiber input is essential to explain Golgi cell responses occurring 7 to 25 ms after a tactile stimulus.

MATERIALS AND METHODS

Surgery and recording procedures

Anesthesia, surgery, and recordings were performed as detailed before (Vos et al. 1999a; Vos et al. 1999b). In brief, 7 male Sprague-Dawley rats weighing 300-400 g were anesthetized with an i.p-injected mixture of ketamine (75 mg/kg) and xylazine (3.9 mg/kg) and supplementary i.m. injections (a third of the initial dose) when required. A homeothermic blanket was used to maintain the core temperature at 37 ºC. The rat's head was fixed in a stereotaxic apparatus and a craniotomy was performed to expose Crus I and II of the left cerebellar hemisphere. Unitary extracellular recordings were made with sharp (1 µm tip) tungsten microelectrodes (± 9.0 MW) lowered individually. Microelectrode signals were amplified and filtered (gain, 5000-10000; band pass 0.4-20kHz) using a multichannel neuronal acquisition processor, digitized and discriminated. Raw and discriminated signals were fed through an auditory monitor for further inspection. Waveforms and recorded spike trains were stored on computer disk for off-line analysis.

Isolated units were identified as Golgi cells using several quantitative criteria: low discharge rates at rest (interspike interval > 20ms) with no bursting, large bipolar spikes of duration > 0.8 ms, long tuning distances (50-150 µm), no complex spikes and location in the granular layer (confirmed by electrolytic lesions at the recording sites).

Stimuli

Facial receptive fields of the Golgi cells were first explored using a cotton-tipped wooden rod. A mechanical stimulation device driven by a Grass 11S stimulator was then used to deliver controlled innocuous mechanical tap stimuli (10 ms) to facial dermatomes at the desired location. The custom-built mechanical device had a 1 mm diameter cylindrical stainless steel probe with flat surface (maximal excursion 2.5 mm) mounted on an electromagnetic activator. Animals were subjected to the mechanical punctate stimulation at 1 Hz. The stimulation protocol consisted of 50 trials without stimulation (a 50 s "OFF period") followed by 200 trials with stimulation (a 200 s "ON period").

Animals were then subjected to a second stimulation paradigm in which a larger area of the face, around and including the point of punctate stimulation (approximately 15 mm2 facial area) was stimulated, using a hand-held brush. Stimulation was presented in blocks of 100 s ("ON period") alternated with 100 s without stimulation ("OFF period"). This second paradigm was used in order to increase the firing rate of the Golgi cells in a smoother way. All stimuli were applied ipsilateral to the recording site.

Data Analysis

Collected spike trains were analyzed off-line using NEX or MATLAB. To characterize Golgi cell firing at rest, the average firing rate (spikes/s) and the coefficient of variation (CV) of the interspike interval (ISI) histogram (standard deviation ISI / mean ISI), were calculated. A smoothed cross-correlation histogram of the spontaneous activity of each pair of Golgi cells was computed from the recorded spike trains at rest. The significance of the correlogram central peak was assessed by calculating the standard Z score, with the critical value for significance set at z>3 (see Vos et al. 1999a and Maex et al. 2000 for details).

The latencies of the evoked responses to mechanical stimulation were measured on fine resolution peristimulus time histogram (PSTHs) (0-500 ms poststimulus; 0.25 ms bin width) constructed using 200 stimulus presentations. Individual response components were isolated as distinct peaks on the PSTH using an algorithm previously described (Vos et al. 1999b). The mean and standard deviation of the isolated peak determined the latency and accuracy of the response.

To quantify the extent of the spike discharge modulation during brush stimulation, a modulation index (referred to as MI) was computed for each Golgi cell: $MI = (FR_{on}-FR_{off}) / (FR_{off}+FR_{on})$, where FR_{off} and FR_{on} were the mean firing rates during the rest periods and the stimulation periods respectively. The mean firing rates were calculated by averaging instantaneous firing rates obtained for successive, non-overlapping blocks of 10sec recording. All pairs of cells recorded were subjected to separate cross-correlation analysis of the "OFF" and "ON" periods of brush stimulation to ascertain whether coordinated activity was modified by the stimulation paradigm. The position of the peaks on the cross correlogram was given by the bin corresponding to the peak maximum, i.e., the highest Z score.

RESULTS

Recordings were obtained from 10 pairs of Golgi cells with each cell of the pair lying on the same parallel fiber beam. The spontaneous activity of isolated units was recorded first. Golgi cells showed low activity at rest (average, 7.74 spikes/s) and a fairly irregular firing pattern (CV average, 0.37). Golgi cells often exhibited large receptive fields, probably reflecting an important excitation by parallel fibers (see Vos et al. 1999b). Most of the simultaneously recorded Golgi cells had overlapping RFs: stimulation of a pair at a particular location evoked responses in both cells.

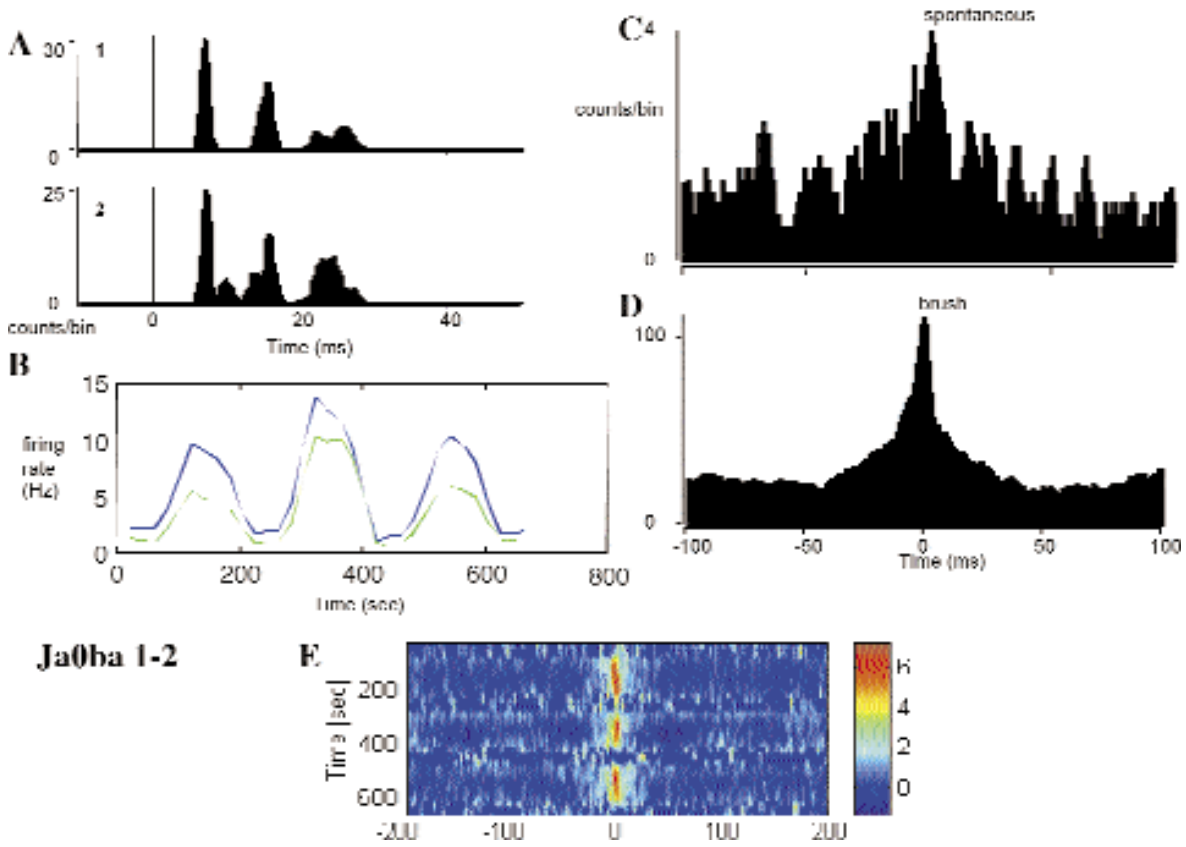

Figure 1

Response of a pair of Golgi cells to the two stimulation paradigms. **A.** Responses to punctate stimulation. Both cells have a short latency early component. **B.** Responses to brush stimulation: the time-course of the average firing rate. Green: cell #1, blue: cell #2. **C.** Cross-correlogram during spontaneous activity. **D.** Cross-correlogram during brush stimulation. **E.** Time course of the cross-correlation using 50s sliding windows incremented every 10s: three periods of brush stimulation are shown. Color code shows Z score.

Responses to punctate stimuli

As previously reported (Vos et al. 1999b; Vos et al. 2000), the evoked responses to short punctate stimuli onto the skin consisted of an early and a late excitatory component. Of particular importance in this study are the differences in latency of the early component, which is due to direct trigeminal input (Morissette and Bower 1996). In some cells (e.g. Fig. 1) the trigeminal component had a short latency, indicative of direct mossy fiber input onto the Golgi cell (Vos et al. 1999b; Vos et al. 2000), in others it had a much longer latency (e.g. cell #3 of Fig. 2) and was due to parallel fiber activation. The late component can consist of many phases, some due to an off response (unpublished observations) and other due to corticopontine inputs (Morissette and Bower 1996; Leergaard et al. 2000).

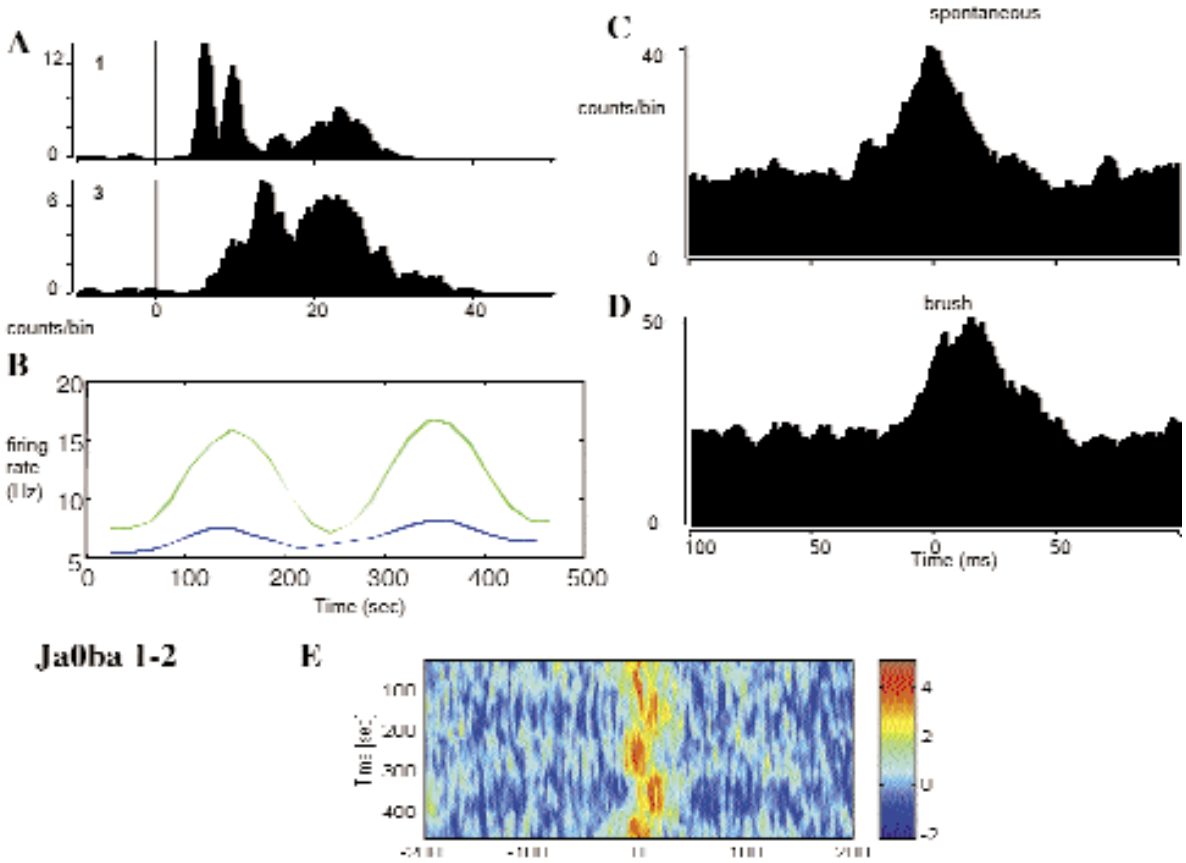

Figure 2

Response of a pair of Golgi cells to the two stimulation paradigms. **A**. Responses to punctate stimulation. Cell #3 has a long latency early component. **B**. Responses to brush stimulation: the time-course of the average firing rate. Green: cell #1, blue: cell #3. **C**. Cross-correlogram during spontaneous activity. **D**. Cross-correlogram during brush stimulation. Notice the shift of the central peak. **E**. Time course of the cross-correlation using 50s sliding windows incremented every 10s: three periods of brush stimulation are shown. Color code shows Z score. The shift of the central peak during stimulation is obvious.

Responses to brush stimuli

The second stimulation paradigm consisted of a peripheral stimulation of a larger area using a manual brush. The stimulus was carefully centered at the locus of the punctate stimulation. Using this protocol we were able to modulate the firing rate of the units in a significant manner: the mean Golgi cell activity increased from 7.8 spikes/s during the resting period to 15.7 spikes/s during the stimulation period (Modulation Index average, 0.36). However, a large disparity was observed between units in the modulation of their discharge rate during paint brush stimulation (MI standard deviation, 0.21). This difference in modulation is exemplified by the pair of Fig. 2.

Responses to punctate and brush stimuli compared

Several measures of the responses of Golgi cells to the two stimulation protocols were compared. We did not find any correlation between the amplitude of the response to punctate stimulation and the modulation index (MI) but a significant positive correlation was found between latency of the punctate response and its MI. Again looking at the pair of Fig. 2 one notes that unit 1 had a short latency double peaked response and was strongly modulated, while unit 3 responded quite late and showed very little firing rate modulation. Fig. 3A shows the highly significant correlation between the difference in modulation index for Golgi cells of a same pair and the difference in latency of the first peak for the two units ($r = 0.891$; $p = 0.0005$).

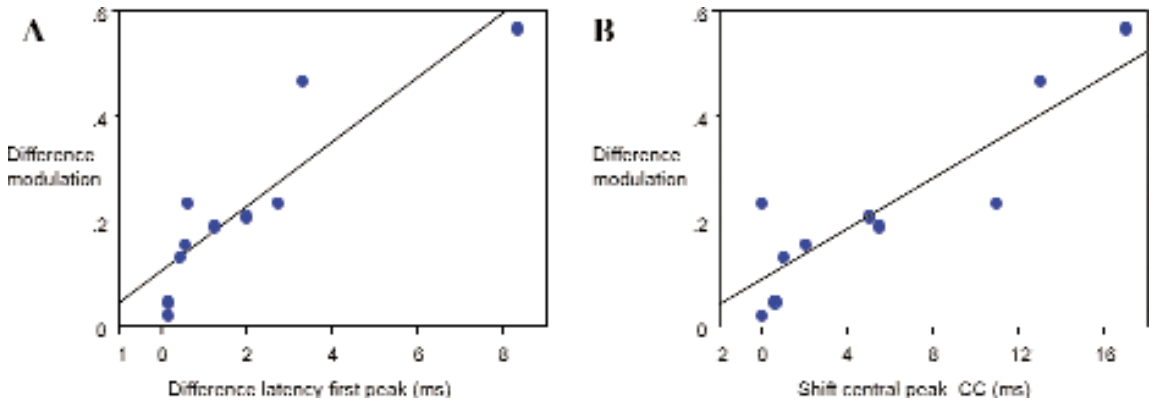

Figure 3

A. Positive correlation between the difference in latency of the first component of the response to punctate stimulation and the difference in firing rate modulation during brush stimulation between the two cells. **B.** Positive correlation between shift of the central peak on the cross-correlogram and the difference in firing rate modulation during brush stimulation between the two cells.

Synchrony during stimulation

As previously reported (Vos et al. 1999a; Maex et al. 2000), parallel fibers synchronized the spontaneous activity of Golgi cells aligned along the length axis of a folium (the parallel fiber axis): all pairs of simultaneously recorded units showed high levels of synchronization at rest (mean Z score, 6.65).

The positive correlation between firing rate and coherence strength predicted by our network model (Maex and De Schutter 1998) was confirmed using the brush stimulation paradigm. Modulation of the discharge activity during stimulation led to an average 40.7% increase in the height of the central peak. An example of this behavior is shown in the pair of Fig. 1: the firing rate of both units and their synchrony increased significantly during the brush stimulation compared to rest. In such cases there was also a significant decrease of the width of the central peak from a mean of 22.3 ms to 10.0 ms, as predicted by the model (Maex and De Schutter 1998; Maex et al. 2000).

In pairs where one of the units did not strongly increase its firing rate during brush stimulation (Fig. 2), the brush stimulation resulted in a shift of the central peak. Sometimes a small peak could be observed at 0 ms, riding on top the main peak. The shift occurred to the right by up to 17 ms in cross correlograms with the strongly modulated unit as reference. Notice that no shift was observed for pairs were both units modulated strongly (e.g. Fig. 1). Indeed, the shift was correlated positively with the difference in modulation between the two units ($r = 0.759$; $p = 0.0149$; Fig. 3B).

DISCUSSION

This experimental study demonstrates that Golgi cell firing rate modulates their synchronization and that the type of excitatory input received plays an important role in their response profiles.

Golgi cells presumed to receive direct mossy fiber excitation (Vos et al. 1999b; Vos et al. 2000) increased their firing rate much more strongly during the brush stimulation (Fig. 3A) then those receiving parallel fiber excitation although their responses to punctate stimulation have only small differences in amplitude. This difference in firing rate modulation could be due to a filtering of the mossy fiber input by granule cells, resulting in a comparative low parallel fiber signal or to transmission failure in the parallel fibers at higher firing rates. The strong response to punctate stimulation makes it unlikely that differences between mossy fiber versus parallel fiber synapses onto Golgi cells, whether in strength or numbers, play a large role. Though we have no supporting evidence, it seems likely that Golgi cell inhibition plays a role in the proposed filtering by the granule cells.

While all pairs showed an increase in synchronization as predicted by our previous modeling studies (Maex and De Schutter 1998; Maex et al. 2000), there was a large difference depending on whether both cells received mossy fiber input or not. When both cells received mossy fiber input the central peak increased in amplitude and decreased in width (Fig. 1). The model predicts that this is due to common parallel fiber input. On electrophysiological basis we cannot exclude common mossy fiber input as a cause, but based on the known branching patterns of mossy fibers we consider it unlikely that Golgi cells separated by more than 1 mm along the parallel fiber beam would receive input from the same mossy fibers. When one of the Golgi cells is excited exclusively through parallel fiber synapses we observed a shift of the central peak (Fig. 2). A separate modeling study has demonstrated that this can be explained by the transmission delays along the parallel fiber system.

Finally, this study provides further insights into the temporal complexity of Golgi cell firing. We have previously suggested that Golgi cells control the timing of granule cell spiking (Maex and De Schutter 1998), resulting in a control over the temporal coding of parallel fiber signals (De Schutter et al. 2000; De Schutter and Bjaalie 2001). Our study also demonstrates that parallel fibers are effective in stimulating Golgi cells. It remains to be explained why the same inputs fail to activate the corresponding Purkinje cells (Bower and Woolston 1983).

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PART 1: Molecular Pharmacology of Angiotensin II AT1 Receptor Antagonists.

Introduction

Angiotensin II, the major peptide hormone of the renin-angiotensin system exerts most of its biological actions by stimulating angiotensin II receptors of the AT_1 subtype. Among these actions, the increased growth and contractility of vascular smooth muscle cells and cardiac myocytes are considered to play a major role in the hypertensive effect of angiotensin II. It may also act on sympathetic nerve terminals to facilitate the release norepinephrine and on the adrenal medulla to increase catecholamine release (Reid, 1992). Angiotensin II has also several physiological effects that result from its interaction with AT_1 receptors in the brain (Saavedra JM, 1999). Among them, angiotensin II may produce a pressor response by increasing the sympathetic activity. AT_2 receptors constitute the other major angiotensin II receptor subtype (Bumpus et al., 1991, Mukojama et al., 1993; Kambayashi et al., 1993). They are expressed in foetal tissues and, in adulthood, they may also participate in blood pressure regulation albeit to a lesser extent as the AT_1 receptors.

During the last decade, much effort has been spend in developing nonpeptide antagonists for the AT_1 receptor for the clinical treatment of hypertension and congestive heart failure (Vallotton et al., 1987). In pre-clinical studies, these antagonists are routinely tested for their ability to affect angiotensin II dose- contractile response curves of vascular tissues such as rabbit aortic rings/strips, a system with very small receptor reserve (Zhang et al., 1993; Robertson, 1998), and of rat portal veins. Hitherto, these assays have constituted the major procedure for comparing the AT_1 receptor binding properties of one antagonist to another. Based on their different capabilities to depress the maximal contractile response to angiotensin II, the antagonists are commonly divided in two categories.

Surmountable antagonists only produce parallel rightward shifts of the dose-response curve without depressing the maximal response. Losartan is the prototype of these antagonists. However, most of the AT_1 receptor antagonists are also capable of depressing the maximal response to angiotensin II. They are denoted as insurmountable. The extent by which these antagonists depress the maximal response to angiotensin II is highly variable; it is almost complete for candesartan but only partial for the antagonists such as irbesartan, valsartan and EXP3174 (the active metabolite of losartan) (Liu et al., 1992; Criscione et al., 1993; Cazaubon et al., 1993; Noda et al., 1993; Mochizuki et al., 1993). Several theories have been put forward to explain the differences in behaviour of the AT_1 receptor antagonists at the molecular level. They include non-competitive antagonist action due to the presence of allosteric antagonist binding sites on the receptor (Wienen et al., 1992), antagonist- mediated conformational changes of the receptor that render them refractory to stimulation (de Chaffoy de Courcelles et al., 1986; Robertson et al., 1994), slow dissociation of the antagonist-receptor complex or even irreversible antagonist binding (Dickinson et al., 1994; Wienen et al., 1993; Olins et al., 1994; Aiyar et al., 1995; Cirillo et al., 1995; De Arriba et al., 1996; Ojima et al., 1997), slow removal of the antagonist from tissue compartments, cells or matrix surrounding the receptor (Robertson

et al., 1992; Panek et al., 1995), coexistence of different receptor subpopulations (Zhang et al., 1993) and the ability of the antagonist to affect the internalisation of AT_1 receptors within the cell (Liu et al., 1992).

Molecular action mechanism of AT_1 receptor antagonists.

A similar discrimination between AT_1 receptor antagonists can also be made by investigating their effect on angiotensin II-mediated responses in intact cell systems but, so far, only limited attention has been spend to this approach. Yet, there are major technical advantages such as the ability to measure direct [^{3}H]-antagonist binding and inhibition of agonist induced responses under identical experimental conditions (Fierens et al., 1999a). In this context, Chinese Hamster Ovary cells have been permanently transfected with the gene coding for the human AT_1 receptor (CHO-hAT1 cells) (Vanderheyden et al., 1999). These cells express human AT_1 receptors at their surface and their intensive investigation in our laboratory has shed new light on the molecular action mechanism of nonpeptide AT_1 receptor antagonists. The major findings can be summarised as follows:

a) When a pre-incubation step with the antagonist is included, surmountable and insurmountable antagonists can be discriminated from each other by measuring the angiotensin II-mediated IP production in CHO-hAT1 cells. Interestingly, it was already noticed by Criscione et al. (1993) that valsartan, an insurmountable antagonist in contraction studies, was unable to depress the maximal angiotensin II- induced aldosterone release in bovine adrenal glomerulosa cells. These latter experiments were done under co-incubation conditions. Similarly, co-incubation with candesartan, EXP3174 and irbesartan only produced parallel rightward shifts of the angiotensin II dose-response curves in CHO-hAT1 cells (Fierens et al., 1999b). Therefore, it appears that all AT_1 receptor antagonists are competitive with respect to angiotensin II. Furthermore we have experimental data that indicate that the investigated antagonists bind to a common or overlapping site on the receptor in a mutually exclusive way (Vanderheyden et al., 2000a). The ability of certain antagonists to decrease the maximal effect of angiotensin II is therefore the result of the experimental set-up (i.e. antagonist pre-incubation) and should not be ascribed to non-competitive behaviour.

b) Radioligand binding experiments reveal that AT_1 receptor antagonists are able to dissociate from their receptor. Hence, irreversible binding can be excluded as an explanation for insurmountable antagonism. Instead, insurmountable inhibition is merely related to the slow dissociation rate of the antagonist- receptor complex: i.e. the blockade is sufficiently long lasting to prevent the access of the receptors to subsequently added angiotensin II. In contrast, the effect of surmountable antagonists such as losartan is very brief. Direct (radioligand dissociation binding) and indirect (restoration of angiotensin II- mediated responses after antagonist preincubation) estimations of the half-life of antagonist- receptor complexes in CHO-hAT1 cells are 7 min for irbesartan, 17 min for valsartan, 30 min for EXP3174 and 120 min for candesartan (Fierens et al., 1999a; Vanderheyden et al., 2000a,b; Verheijen et al., 2000).

c) Most insurmountable AT_1 receptor antagonists do not produce a complete decrease of the maximal angiotensin II- mediated response in systems as diverse as rabbit aortic strips and CHO-hAT1 cells (Vanderheyden et al., 1999; Fierens et al., 1999b). The maximal response in CHO-hAT1 cells decreases by no more than 94 % for candesartan, 70 % for EXP3174, 30 % for irbesartan and by less than the level of detection for losartan. It was therefore proposed that antagonist - AT_1 receptor complexes may adopt two distinct states: a fast reversible state that accounts for the surmountable inhibition, and a tight binding state that accounts for the insurmountable inhibition (Fierens et al., 1999b). A close fit was found between the experimental data and computer- simulated curves according to a two-step, two-state model in which the equilibrium between both two states is dictated by the chemical nature of the antagonist (Vauquelin et al., 2000a,b). Recent site- directed mutagenesis studies, involving the substitution of basic amino acids of the AT_1 receptor into neutral ones, revealed that Lysine199 plays an important role for the slow reversible antagonist binding (Fierens et al., 2000a).

d) Ligand molecules that are dissociated from their receptors may accumulate and, if not constantly removed, they may bind again. This "reassociation" or "rebinding" of the antagonist to the receptor may increase the duration of the blockade. Indeed, in candesartan- pre-treated CHO-hAT1 cells, the recovery of functional AT_1 receptors was half-maximal after 2 hours when rebinding was prevented and only after 6 to 8 hours when rebinding was allowed to take place (Vanderheyden et al., 1999, 2000a; Fierens et al., 1999b).

Relevance of the *in vitro* binding properties of AT1 receptor antagonists.

From the antagonists tested by us to date, binding of candesartan to the human AT_1 receptor in CHO-hAT1 cells displays unmatched longevity. At least for CHO-hAT1 cells, its antagonistic action is further prolonged by "rebinding" phenomena. A link between this in vitro property of cansesartan and its long lasting anti-hypertensive effect (Sever et al., 1997, Lacourcière et al., 1999) is plausible but remains to be established. Yet, in vivo studies on rat (Morsing et al., 1999) and a clinical study on human (Delacrétaz et al., 1995) clearly show that the anti-hypertensive action of candesartan remains when its plasma concentration has already declined substantially. On the other hand, despite its relatively fast dissociation from the receptor and the absence of substantial rebinding in CHO-hAT1 cells (Vanderheyden et al., 2000b), irbesartan is also recognised to have a long lasting anti-hypertensive effect (Oparil, 2000). For this antagonist, it is not known whether factors else than its plasma half-life contribute to its duration of action.

The molecular- pharmacological studies on CHO-hAT1 cells pointed out that the terms "surmountable" and "insurmountable" are only appropriate under particular experimental conditions (i.e. involving antagonist pre-treatment). The discrimination between surmountable and insurmountable AT_1 receptor antagonists as well as their potential for rebinding is, in fact, related to more fundamental properties of the antagonist- receptor interaction: i.e. their affinity and the stability (half-life) of the complexes. More information on the different factors that affect the duration of the *in vivo* anti-hypertensive effect of AT_1 receptor antagonists is clearly needed befo-

re transposing conclusions from studies at the receptor level to clinical situations. In this respect, the present day's large variety in pharmacological models permits the examination of the potential impact of various pharmacodynamic and pharmacokinetic factors on systems with gradually increasing complexity. In this project, we propose the following experiments with AT_1 receptor antagonists on CHO-hAT1 cells as well as on more complex systems:

- To further complete and investigate the structure-activity relationship of the nonpeptide AT_1 receptor antagonists we set out to test telmisartan and eprosartan on CHO-hAT1 cells on the pharmacological models described below.
- Radioligand binding (with special emphasis to rebinding and non-specific accumulation) and functional studies on different human cell types endogenously expressing the AT_1 receptor, including human vascular smooth muscle cells.
- Comparison between antagonist actions on native cells and on cells in which the AT_1 receptors were partially desensitised by chronic exposure to angiotensin II.

a) Effect of additional AT_1 receptor antagonists.

The AT_1 receptor binding properties of candesartan, EXP3174, valsartan, irbesartan and losartan have already been tested on CHO-hAT1 cells. These antagonists are all biphenyl-tetrazole derivatives. In addition, the AT_1 receptor binding properties were also tested for LY301875 and LY303336, two polysubstituted 4-aminoimidazole derivatives. This difference in structure does not affect the ability of LY301875 and LY303336 to undergo long- lasting binding to the AT_1 receptors (and, hence, to produce insurmountable antagonism) but it may explain the overall lower affinity of these compounds for the receptor. Meanwhile, two new AT_1 receptor antagonists, telmisartan and eprosartan , have become available for the treatment of hypertension (Oparil, 2000). Whereas telmisartan is a biphenyl-tetrazole derivative, eprosartan has a different overall structure. To further explore differences and/or similarities between biphenyl-tetrazole derived and structurally different AT_1 receptor antagonists, we will also investigate the binding and antagonistic properties of telmisartan and eprosartan on CHO-hAT1 cells. Special attention will be focussed on their overall affinity (competition binding with [^{3}H]candesartan and [^{3}H]angiotensin II, Vanderheyden et al., 1999; Fierens et al., 1999a), their competitivity (effect on angiotensin II dose-response curves under co-incubation conditions, Fierens et l., 1999b), the ratio between fast reversible and tight binding (inhibition curves of the angiotensin II- mediated IP production, Fierens et al., 1999b), their dissociation rate (indirectly by investigating the rate of restoration of angiotensin II- mediated IP production and [^{3}H]candesartan binding in antagonist-pre-treated cells, Vanderheyden et al., 2000a) and the repercussion of rebinding on the duration of the antagonistic effect (Fierens et al., 1999a; Vanderheyden et al., 2000a).

CHO-hAT1 cells have been transiently transfected with genes coding for mutated human AT_1 receptors (collaboration with Prof. L. Hunyady, Budapest). These mutation studies have shed light on the important role of Lysine199 for the slow reversible antagonist binding (Fierens et al., 2000a). CHO cells expressing mutated human AT_1 receptors in which Lysine199 and other basic amino acids are individually substituted by neutral ones will therefore also be used to further explore the differences and/or similarities between telmisartan, eprosartan and the alrea-

dy tested biphenyl-tetrazole derived AT_1 receptor antagonists.

b) Radioligand binding and functional studies on human cell types endogenously expressing the AT_1 receptor.

CHO-hAT1 cells are highly artificial since they express the human AT_1 receptor in cells from another species. The possibility therefore remains that the antagonist binding and rebinding properties in CHO-hAT1 cells are atypical for the human AT_1 receptor. In contrast, human cell lines that endogenously express the AT_1 receptor offer the advantage that they do not constitute synthetic constructs. Some of them may also produce an extracellular matrix similar to that for smooth muscle cells in vascular walls. Finally, the receptor concentration is physiologically more relevant.. In collaboration with Dr. S. Bottari (Grenoble, France) we can have access to NCI-H295 cells (adrenal medulla), HVSMC cells (vascular smooth muscle), AT_1-receptor- containing NG108-15 and NIE-115 cells (neuronal) and AT_1- as well as AT_2 receptor- containing L6C5 cells (skeletal muscle). The most relevant experiments that were performed on CHO-hAT1 cells will be repeated for the other cell types. We are especially interested in:

- The proportion between loose and tight antagonist binding (i.e. the proportion between surmountable and insurmountable inhibition).
- The half-life of the tightly bound antagonist-receptor complex
- Rebinding to the receptor and the existence of additional non- receptor binding sites which are responsible for the accumulation of antagonist molecules in the vicinity of the receptor.

The phenomenon of "rebinding" or "reassociation" has been well described by Limbird et al. (1996). When not quickly removed, ligands that are released from their receptor will start to accumulate in the medium and, provided that their affinity is sufficiently high, a substantial portion of those released ligands may bind again. Such continuously recycling has been held responsible for prolonging the binding/effect of AT_1 receptor antagonists such as candesartan and EXP3174 in washout experiments on CHO-hAT1 cells (Fierens et al., 1999a, Vanderheyden et al., 2000a). It may also contribute to the long-lasting AT_1 receptor blockade by insurmountable antagonists such as candesartan in aortic strip and portal vein contraction experiments (Robertson et al., 1992; Ojima et al., 1997; Morsing et al., 1999) as well as to its long-lasting *in vivo* anti-hypertensive action.

Alternative causes for a local build-up of the antagonist concentration near the AT_1 receptor were evoked by Panek et al. (1995). These authors found that certain insurmountable antagonists (CI-996 and SKF108834) were rapidly dissociating in radioligand binding studies but had a long- lasting action in contraction studies on rabbit aorta. Moreover, the time lag after which the response was restored in washout experiments appeared to be dependent on the initial antagonist concentration. These data suggest that the long-lasting action of certain antagonists is not necessarily due to their slow dissociation from the receptor but may be related to their slow removal from compartments within tissue, cells or matrix surrounding the AT_1 receptor. This may be related to factors such as lipid solubility, receptor environment and kinetics of distribution and metabolism of the antagonist within the tissue (Panek et al., 1995; Robertson

et al., 1992). At present, no information is available about the factors that might potentially affect the local accumulation of AT_1 receptor antagonists and, in particular, whether rebinding requires a special organisation of the AT_1 receptor (e.g. presence in clusters) to take place.

The following experimental approach is proposed. For the receptor-binding characteristics, we will investigate the antagonistic profiles of candesartan, EXP3174, telmisartan, eprosartan, irbesartan and losartan by functional studies. Besides measuring the antagonist's effects on the angiotensin II- mediated IP production (Table 1a), these cells also offer the possibility of measuring more distant and relevant functional responses (Table 1b). Provided that the receptor concentration is sufficiently high, direct binding of [^{3}H]candesartan, [^{3}H]valsartan and [^{3}H]irbesartan can be investigated as well (Table 1c). If not, indirect information can be obtained by competition and kinetic (i.e. delay of the association rate in antagonist- pre-treated cells, Hara et al., 1995; Vanderheyden et al., 2000a) experiments using [^{125}I]sarile.

In addition, certain of the above mentioned cell lines may offer the possibility to investigate the effect of antagonists on angiotensin- mediated cell proliferation and differentiation. Stimulation of AT_1 receptors will induce the proliferation of vascular smooth muscle cells by a protein kinase C- dependent pathway (by activation of mitogen-activated protein kinases) as well as by an independent pathway (by activation of tyrosine kinases such as Scr, Jak en Fak) (Marrero et al., 1995; Berk, 1999). In contrast, stimulation of AT_2 receptors will inhibit cell differentiation and cell growth (Schmitz et al., 1997). The L6C5 cell line derived from skeletal muscle possesses both AT_1 and AT_2 receptors and it has been shown to constitute an excellent model to investigate the effect of both receptor subtypes on cell differentiation (detected by the fusion of myoblasts en myotubules and induced by insulin like growth factor 1 (IGF-1)) (Bottari S.P. et al., personal communication). This cell line offers therefore the possibility to investigate, on the same experimental system, the ability of AT_1 receptor antagonists to shift a predominantly AT_1 receptor response to angiotensin II into an AT_2 receptor response.

Table 1

i) Inositol phosphate production

Goal	experimental approach
AT_1 receptor activity	angiotensin II concentration-response curve
Insurmountable inhibition	antagonist inhibition curve (preincubation versus co-incubation with angiotensin II)
Kinetic parameters	delay in response to angiotensin II in washout experiments (with losartan)
Rebinding	same, without losartan

ii) More distant functional responses.

Cell type	response
NCI-H295 (adrenal medulla)	aldosterone secretion (measured by RIA)
HVSMC (vascular smooth muscle)	cell growth ([^{3}H]thymidine en BrdU incorporation)
L6C5 (skeletal muscle)	inhibition of cell differentiation (induced by IGF-1)
NG108-15 and NIE-115 (neuronal)	[^{3}H]noradenalin secretion

iii) Radioligand binding

Goal	experimental approach
General characterisation	saturation and competition binding
Kinetic parameters	association and dissociation rates
Rebinding	effect of losartan on dissociation rate
Tissue accumulation	non-specific binding
Internalisation	determination of acid-sensitive and Resistant binding (for [^{3}H]angiotensin II)
Desensitisation	[^{3}H]antagonist binding in angiotensin II-pre-treated cells

c) Effect of chronic exposure of AT_1 receptors to angiotensin II on antagonist action.

In contrast to the hitherto performed studies on CHO-hAT1 cells, in which the antagonist is always added to the cells before or along with angiotensin II, the AT_1 receptors in animals or patients have been exposed to angiotensin II prior to the administration of AT_1 receptor antagonists. Similar considerations also hold true for contraction studies on isolated blood vessels and it is even plausible that angiotensin II- mediated receptor desensitisation is a causal factor for the antagonist- mediated increase in their maximal responsiveness to angiotensin II in a number of studies (Robertson et al., 1994; Morsing et al., 1999). In this context it is clinically relevant whether angiotensin II induced internalisation and/or desensitisation influences the antagonist binding properties and their anti-hypertensive action.

Angiotensin II is well known to induce rapid internalisation of the AT_1 receptor in various cells and this process reflects the endocytosis of the agonist-bound receptor (Hein et al., 1997). This internalisation may be part of their recycling process of the receptors (Anderson et al., 1993, Hein et al., 1997). However studies with mutated receptors have pointed out that there is no causal link between receptor internalisation and receptor activation and desensitisation (Hunyadi et al., 1994; Thomas et al., 1995). It has also been advanced that the distinction between surmountable and insurmountable antagonist binding may be related to differences in the subcellular localisation of the antagonist-bound AT_1 receptors (Liu et al., 1992). Recent findings with mutant receptors which lost their ability to internalise (51) as well as with fluorescent labelled receptors (Hein et al., 1997) rather suggest that antagonist- bound receptors remain at the cell surface.

Studies on CHO-hAT1 cells will involve radioligand binding experiments with [^{3}H]angiotensin II to investigate the amount of internalised receptors as a function of the incubation time and the agonist concentration. In these experiments, expertise from previous work will be used to discriminate the binding to cell surface and internalised receptors (Fierens et al 2000b). Following the pre-treatment of the cells with unlabelled angiotensin II, it is possible to monitor the reappearance of recycled AT_1 receptors at the cell surface by binding studies with [^{3}H]candesartan (which associates very fast at 5 nM, Vanderheyden et al., 2000a). Similar experiments involving a double preincubation step (the first one with angiotensin II to induce receptor internalisation and the second one with antagonist) will provide information about the effect of agonist exposure on the extent and duration of antagonist action. For these experiments, it is not possible to monitor the recovery of receptors by measuring the IP production because of its cumulative nature (due to the presence of LiCl in the assay medium). Yet, human cell lines expressing AT_1 receptors allow the measurement of more distant functional responses and, hence, to circumvent the technical limitations that are inherent to IP measurements.

Obviously, it is of interest to explore whether the angiotensin II- mediated internalisation/ desensitisation of AT_1 receptors and its potential effect on antagonist action is cell dependent or not. In this respect, we will also have the opportunity to compare data from our intact cell experiments with those of related contraction studies on rat portal vein by Dr. P. Morsing and co-workers (unpublished observations).

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PART 2: The use of Endothelin 1 in a model for focal transient ischemia : a microdialysis study in rat brain.

Stroke is the third major cause of death, and the main disabling neurologic disease in the industrialized countries. In an ischaemic situation, the brain is deprived of oxygen and glucose, and consequently, the ATP-driven ion pumps fail to work. Hence, energy insufficiency leads to membrane depolarization, which in its turn results in a large release of various neurotransmitters, such as glutamate, dopamine and GABA. In various experimental studies however, it was observed that neuronal death is not occurring immediately after the ischaemic injury. This observation has encouraged the research and development of neuroprotective agents, which would be administered upon stroke, but still be able to prevent further neuronal damage.

In general, this study is situated in the framework of the research for neuroprotective drugs in cerebral ischaemia. Ultimately, its goal is to find a pharmacological treatment, which can mitigate, or even prevent, any neuronal damage following an ischaemic insult.

For this purpose, first, an animal model for studying focal, transient cerebral ischaemia was developed. Since in humans an ischaemic insult is seldom permanent, it was aimed to develop an animal model, incorporating transient occlusion, i.e. with reperfusion. Hence, the clinical situation is mimicked as closely as conceivable with an animal model.

Studying the different animal models described in literature, the Et-1 model was selected. Et-1, an endogenous peptide, is one of the most potent vasoconstrictors known to date. Using this peptide as a tool to constrict one of the main cerebral arteries (i.e. the MCA), creates an animal model for focal transient cerebral ischaemia.

The characterization of this model was done using microdialysis, laser Doppler flowmetry and histology. With microdialysis, the release pattern of extracellular striatal dopamine, glutamate and GABA was monitored. After a dose-response study, a 120 pmol Et-1 dose was selected to conduct further work with. Injecting 120 pmol Et-1 provoked a reproducible ischaemic infarct, with reperfusion incorporated into the design of the model. Laser Doppler studies confirmed the drop in striatal blood flow after the micro-application of Et-1 in the proximity of the MCA, as well as the return to basal blood flow levels, with a certain time delay. Administration of a non selective Et-1 antagonist, locally in the striatum, before Et-1 administration in the proximity of the MCA, resulted in a similar release pattern of the monitored transmitters, compared with the Et-1 only treated animals. On the other hand, when administering the Et-1 antagonist at the same injection site as Et-1, but *beforehand*, an inhibition of the Et-1 effects on extracellular striatal transmitter levels was observed. Hence, this confirmed the hypothesis that Et-1 was active via its receptor sites on the MCA and/or its receptor sites on the MCA's lenticulostriatal branches, resulting in a vasoconstriction, and not by a direct action of Et-1 upon striatal dopaminergic neurons through diffusion.

Histological studies finally showed the existence of a reproducible ischaemic infarct, located in the striatum and expanding to the cortex.

Secondly, the Et-1 animal model was used to test the effect of different potential neuroprotective agents.

Since it is increasingly recognized that the development of highly selective compounds for neuronal Ca^{2+} channels is not only important for the treatment of stroke, but also for further clarification of the mechanisms of neurodegeneration, one of the drug trials dealt with the use of Ca^{2+} antagonists in the Et-1 model. In this study, two commonly used antagonists, nimodipine (0.1 mg/kg) and verapamil (0.4 mg/kg) were tested, next to a relatively new compound, LY393613 (15 mg/kg), a novel neuronal (N/P/Q-type) calcium channel blocker. Nimodipine and verapamil are two L-type calcium channel blockers. Nimodipine was also selected for its known hemodynamic properties. LY393613 was demonstrated to significantly attenuate the ischaemia-induced increases in extracellular glutamate levels, and coherently to attenuate the infarct volume. These effects were shown not to be due to the direct blockade of the vasoconstrictive actions of Et-1, nor to the alterations of cerebral blood flow. It was concluded that LY393613, a calcium channel blocker with low molecular weight, may be useful as an anti-ischaemic agent.

Glutamate and glutamate receptors also play an important role in the acute neurodegeneration, following cerebral ischaemia. The sudden energy failure results in a massive release of excitatory amino acids and other neurotransmitters. Moreover, energy failure prevents the reuptake of glutamate by its transporter, and reversal of the transporter may further increase the extracellular glutamate levels. Performed studies showed that the newly developed kainate receptor antagonist ($iGluR_5$), LY377770, behaves as a neuroprotectant, both in models for global and focal ischaemia. The effects of LY377770, a selective $iGluR_5$ receptor antagonist, MK801, a selective NMDA receptor antagonist, as well as CNQX, a mixed AMPA/kainate receptor antagonist, were tested. The extracellular striatal effluxes of dopamine and glutamate were monitored, after i.p. administration of LY377770 (75 mg/kg), MK801 (2.5 mg/kg) or CNQX (10 mg/kg). Microdialysis studies showed that only the ischaemia-induced glutamate release was significantly affected using LY377770. On the other hand, MK801 was successful in attenuating the dopamine as well as the glutamate efflux, either in the pre- as well as in the post-treated animals. CNQX showed to have only minor effects. Laser Doppler flowmetry showed that none of the tested compounds was able to affect the blood flow significantly. Histology studies showed significant protection with the new compound LY377770, as well as with MK801. Hence, since LY377770 (1) has a good solubility, (2) has only few side effects and (3) protects against ischaemic brain damage, even when administered post-occlusion, it may be useful for the treatment of acute neurodegenerative diseases.

Finally, the free radical formation and its contribution to the development of the ischaemic insult was focused. Free radicals, nitric oxide and especially dopamine and quinones, i.e. dopamine's oxidative products, were shortly described. Quinone formation was measured on-line in the dialysates during development of ischaemia. It was observed that excessive quinone formation occurred shortly after the ischaemia-induced dopamine release had reached its maximum. Hence, it was assumed that the formation of quinones coincided with the reperfusion phase. Increase in striatal quinones started approximately 10 minutes after the Et-1 induced

vasoconstriction, peaked 20 minutes later and normalized to basal levels within 60 minutes after the Et-1 injection. Assuming the significant increase in quinone formation to be indicative for the start of the reperfusion phase, it can be concluded that the selected 120 pmol Et-1 micro-application is able to provoke a temporary and tailored vasoconstriction. MRI (magnetic resonance imaging) studies confirmed reperfusion to occur 11 to 22 minutes after the injection of Et-1 (Steve Laureys, unpublished results).

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The metabolism of inositol phosphates and phosphoinositides in neuronal cells

The metabolism of inositol phosphates and phosphoinositides is particularly important in brain and is implicated in various signaling cascades involving inositol trisphosphate, inositol tetrakisphosphate and phosphatidylinositol trisphosphate. Those molecules are second messengers in various cell events such as cell proliferation, apoptosis or Ca^{2+} dependent control mechanisms and Ca^{2+} itself. They are controlled by various members of the inositol 5-phosphatase family and 3-kinase family (Erneux et al., 1998).

Cloning a cDNA encoding human inositol trisphosphate isoenzyme C (Dewaste et al., 2000)

Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) 3-kinases are enzymes which catalyse the phosphorylation of Ins(1,4,5)P_3 to inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4), both potential modulators of calcium homeostasis (Berridge and Irvine, 1989). Data presented by Irvine and colleagues indicated that Ins(1,3,4,5)P_4 binds to a protein of the GAP1 family and thereby modulates Ca^{2+} mobilisation by Ins(1,4,5)P_3 (Cullen et al. 1995). Another indication of a key regulatory role for the Ins(1,4,5)P_3 3-kinase(s) comes from recent data indicating that Ins(1,3,4,5)P_4 controls the frequency of calcium oscillations in HeLa cells (Zhu et al., 2000). cDNAs encoding two 3-kinase isoenzymes have been reported to be present in rat and in human (referred to as Ins(1,4,5)P_3 3-kinases A and B) and these have been expressed in bacteria or in COS-7 cells (Takazawa et al., 1990 and 1991). 3-kinase A was shown to be expressed at high levels in the hippocampal CA1 pyramidal and in Purkinje cells (Mailleux et al., 1991). Recent data obtained in our laboratory have shown that Ins(1,4,5)P_3 3-kinase B is expressed in astrocytes and is phosphorylated in response to purinergic agonists (Communi et al., 1999).

In general, Ca^{2+} in a complex with calmodulin stimulates mammalian Ins(1,4,5)P_3 3-kinase activity despite the presence of proteolytic fragments often obtained during extraction and purification of the enzyme (Takazawa et al., 1989). This stimulating effect was attributed to the presence of a calmodulin binding domain at the N-terminal end of the protein (Takazawa and Erneux, 1991). However, there is considerable cell-to-cell variability in the sensitivity of Ins(1,4,5)P_3 3-kinase activity to Ca^{2+}/calmodulin activation. This could result from the existence of different isoforms and/or splice variants of a single gene. This difference in sensitivity of Ca^{2+} was indeed also shown for the two previously reported Ins(1,4,5)P_3 3-kinase A and B isoforms when expressed in E. coli: the A isoform was stimulated two to three fold by Ca^{2+}/calmodulin whereas the B isoform was stimulated up to 10 fold (Takazawa et al., 1990 and 1991). The specificity of Ins(1,4,5)P_3 phosphorylation by recombinant 3-kinases A and B at the 3-position of the inositol ring has been resolved by HPLC (Craxton et al., 1994).

Recently, a new family of inositol polyphosphate kinases has been reported and several cDNAs encoding these enzymes have been cloned: three of them (KIAA0263, PiUS and KCS1) are inositol hexakisphosphate (InsP_6) kinases (Voglmaier et al., 1996 and Schell et al., 1999) while ArgRIII (also known as Arg82) was shown to be a multifunctional kinase (IPMK) which phosphorylates Ins(1,4,5)P_3 and Ins(1,4,5,6)P_4 in *Saccharomyces cerevisiae* (Saiardi et al., 1999). *In vitro*, ArgRIII catalyses the phosphorylation of Ins(1,4,5)P_3 at both the 3- and 6-posi-

tion forming Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4. A role of this multikinase in nuclear mRNA export was recently described (Saiardi et al., 2000). None of these enzymes appears to specifically produce Ins(1,3,4,5)P_4 as shown for the A and B forms of mammalian Ins(1,4,5)P_3 3-kinases. In mammalian systems, no phosphorylation at the 6-position (which would form Ins(1,4,5,6)P_4) has ever been observed.

There are hints in the literature that the family of Ins(1,4,5)P_3 3-kinases may be more diverse. In human platelets, rat thymus or thyroid cells, the Ins(1,4,5)P_3 3-kinase appears to run on SDS gels as higher Mr protein(s) as compared to 3-kinases A and B. We have now reported the sequence and expression of a third mammalian Ins(1,4,5)P_3 3-kinase (3-kinase C) that demonstrates enzymatic activity when expressed in *E. coli* or COS-7 cells. This enzyme is rather ubiquitous based on Northern blot analysis. We show for the first time an inhibition of activity by calcium measured on transfected COS-7 cells suggesting a different mode of Ins(1,3,4,5)P_4 production in intact cells as compared to the A and B isoforms expressing cells. Depending on the isoenzyme expression, Ins(1,3,4,5)P_4 production in various cells is tightly controlled (Dewaste et al., 2000).

Identification of SHIP2 in Neurospheres (Muraille et al., submitted)

In 1997, we reported the cloning of a cDNA encoding human polyphosphatase 5-phosphatase SHIP2 (Pesesse et al., 1997). SHIP2 contains one N-terminal SH2 domain, a catalytic 5-phosphatase domain in the central part , proline-rich regions, a NPXY motif, and a C-terminal SAM domain. By Northern and Western blotting SHIP2 was demonstrated to be expressed in different region of mouse brain (cortex, cerebellum, astrocytes...) (Muraille et al., 1999 ; Kudo et al., 2000).

We conducted a study in collaboration with Prof Shiffmann (Laboratory of Neurophysiology, ULB) in order to map the SHIP2 in brain. We demonstrated that SHIP2 mRNA was highly expressed in the ventricular zone at early embryonic stages and subventricular zones at latter stages of brain and spinal cord and in the sympathetic chain .No expression was seen in differentiated fields. The restricted expression was maintained from E11.5 to birth. In the periphery, large expression was detected in muscle and kidney and moderate expression in thyroid, pituitary gland, digestive system and bone. In the adult brain, SHIP2 was mainly restricted in structures containing neural stem cells such as the anterior subventricular zones and the rostral migratory stream and the olfactory tubercle. We have shown directly by Western blotting using a SHIP2 antibody the expression of the protein in proliferating neurospheres in culture in contrast to non proliferating differentiated neurosheres (in collaboration with Bernard Rogister). The data therefore suggest a role of SHIP2 in proliferating rather than in differentiating events (Muraille et al., submitted).

The protein partners of SHIP2 in brain (identification by a yeast two hybrid screening in human brain)

By these different domains, SHIP2 is able to interact with different proteins and to organize the cellular response to stimuli. By its SH2 domain, SHIP2 binds phosphorylated tyrosins of the ITIM (immunoreceptor tyrosine-based inhibitor) motif of FcγRIIB, inhibiting the B lymphocytes

activation in the immune response (Muraille et al., 2000). By the same domain, SHIP2 binds Shc, inhibiting the association with Grb2-Sos and activation of the Ras pathway (Ishihara et al., 1999). SHIP2 is phosphorylated constitutively by $p210^{bcr/abl}$ in progenitor cells of chronic promyelocytic leukemia and could play a role in myeloid expansion (Wisniewski et al., 1999). We have studied the potential other protein partners of SHIP2 by the yeast two-hybrid system.

In a two-hybrid assay, the cDNA of the proteins of interest and the cDNA library are cloned into separate vectors, one that generates a hybrid protein with the GAL4 activating domain and one that generates a hybrid protein with the GAL4 DNA-binding domain. Both domains are required to reconstitute a functional GAL4 transcriptional activator. The two hybrid plasmids are then cotransformed into a yeast host strain which contains in its genome selection genes under the control of a GAL4 dependent promoter. Only the transformants of the library in which the target finds a partner, and thus brings together the two GAL4 functional domains, will be positive for expression of the selection gene (Fields and Song, 1989). The proteins can then be isolated, cloned, sequenced, identified and produced in vitro for further investigation. This technique proposed by Fields and Song, has since been successfully used in a large number of studies. Numerous two-hybrid works helped for example to better understand Alzheimer's disease (AD) (Imahori K et al., 1997), and to identify PDZ containing protein partners which are very important in the neurons receptor targeting. This technique in molecular biology has led to an increased understanding of disease processes that affect the brain and to novel forms of therapy.

Our SHIP2 partners research consisted in the screening of a human adult brain cDNA library with the SHIP2 proline-rich region. From 600000 transformants, 62 clones were selected by nutritional tests and 46 of them were specific of the bait tested. We characterized among these potential partners the JNK-interacting proteion or Jip 1, Insulin receptor substrate protein or IRSp53, Vinexin and Intersectin proteins, all expressed in brain, and respectively implicated in the insulin or EGF transduction pathways or in endocytic/exocytic processes. Only one of them interacts also with SHIP1 indicating therefore that SHIP1 and SHIP2 for the other partners have different proline rich domains as shown in our hands in a yeast two hybrid screening assay. These partners are now under biochemical studies to assess if they are physiologically relevant.

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VESTIBULAR COMPENSATION, A MODEL OF BRAIN PLASTICITY

Background

Behavioural level

In mammals, unilateral labyrinthectomy causes immediate and dramatic disorders. The most prominent static symptoms combine a spontaneous nystagmus with its slow phases directed to the side of injury, a lateral deviation of the head and body toward the same side and a tilt of head and neck in the frontal plane, also toward the side of injury. However, the nervous system responds to this lesion in such a manner as to generate an efficient postural compensation. This form of brain plasticity and of motor learning, called vestibular compensation, is an interesting model because vestibular damage can be inflicted in a very precise manner and because the deficiencies that follow and the steps in their compensation are uniform from one animal to the next. Static vestibular compensation is rapid (2 or 3 days in the guinea pig). It has been suggested with some experimental support in decerebrate or anaesthetized animals that the static symptoms subsequent to a unilateral labyrinthectomy were due to a loss of resting activity in the neurones in the ipsilateral vestibular nuclei, whereas static vestibular compensation was due to a restoration of that resting activity.

At the neuronal level

The labyrinth is essential for our balance. It detects the movements of the head and the position of the head in space. When ciliar cells in the labyrinth are stimulated, they excite the vestibular nerve fibres which, in turn, stimulate the secondary vestibular neurones in the ipsilateral vestibular nuclei. The activation of the vestibular nuclei causes a deviation of the eyes, head and body towards the opposite side. At rest, in the absence of any head movement, vestibular fibres are not silent. In the guinea pig, their mean basal discharge is 60/sec. However, the balance is maintained because both vestibular nerves are spontaneously active. In previous years, we have studied the behaviour of the secondary vestibular neurones in awake control guinea pigs and in awake animals labyrinthectomized beforehand. In control animals, the mean firing rate of the vestibular neurones is 36/sec and none of them is silent. Just after a unilateral labyrinthectomy, it decreases to 7/sec and 75 % of the neurones are silent. However, this is followed by a restoration of the spontaneous activity of the vestibular neurones which starts 12 h after the lesion and is complete 1 week later. Our laboratory now focus its attention on the mechanisms of this spectacular recovery. This restoration of activity occurs at the same velocity after a bilateral labyrinthectomy when there is no behavioural error signal. This seems to indicate that the recovery mechanism take place in the neurones deprived of their labyrinthine input themselves.

Works carried out in 2000

1. We have compared the pacemaker activity of the neurones in the left medial vestibular nucleus of control guinea pigs with that of animals labyrinthectomized one week beforehand. For this purpose, the endogous (pacemaker) activity of the neurones was assessed by their spontaneous activity recorded extracellularly in brain stem slices in presence of a cocktail of neurotransmitters blockers (CNQX, dAPV, bicuculline and strychnine) which freed them from their main synaptic influences. The left medial vestibular nucleus (MVN) was explored in a very systematic way. In presence of neurotransmitter antagonists, we found that the mean number of spontaneously active neurones detected in a single MVN by our procedure increased dramatically from 9 in slices from control guinea pigs to 26 in slices from animals labyrinthectomized on the left side one week beforehand. The results show that deprivation of the vestibular neurones from their labyrinthine input causes a change in the deprived neurones themselves. They suggest that an increase in pacemaker activity might be one of the factors responsible for the restoration of spontaneous activity in the vestibular neurones after labyrinthectomy.

2. In the course of an attempt to eliminate synaptic influences, we submitted the brain stem slices to a perfusion containing a low Ca^{++} - high Mg^{++}. We found that this medium induced a class of neurones to fire in bursts. It was further proved that the calcium channels types R, P, N were involved in determining the firing pattern of the vestibular neurones (Neuroscience Letters, 2000).

3. We have explored the responses in firing rate of the vestibular neurones to different current stimulations. Such a purpose necessitated to perform intracellular stimulation and recording of vestibular neurones in brain stem slices. This research has been very successfull. It was found that after vestibular compensation, the response of the neurones to dynamic stimuli was increased. In other words, we have found a new type of neuronal plasticity. In response to the suppression of one of its major synaptic inputs, vestibular neurones adapt themselves by modifying the number and types of electroreceptors in their membrane.

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OTHER PUBLICATION

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Genetic definition of brain tumours by analysis of their genome using microsatellites

Introduction

Brain tumours are often associated with bad clinical prognosis. Their Treatment lacks efficiency, and their histopathological diagnosis misses easy and reproducible criteria. The latter may be partly due to the histological heterogeneity within and in between these tumours. There is obviously a need for neuro-oncology to have better tools for establishing diagnostic and prognostic criteria of brain tumours. This would also allow better treatment trials.

Our ongoing genetic study of brain tumours has a dual goal:

1. A clinical aim: to correlate genetic profiles of brain tumours to a diagnosis, a prognosis and a therapeutic response.
2. A physiopathological aim: to define small enough altered chromosomal region(s) associated with specific brain tumour sub-groups. This would allow to identify tumour suppressor genes and/or oncogenes involved in tumorogenesis.

Currently, we are studying oligodendrogliomas and ependymomas. In both studies, tumours have first been retrieved from formaline- fixed and paraffin-embedded archival tissues from three main institutions: St-Luc (Brussels), Institute of Neurology (London) and Hopital R. Salingro (Lille). We are using microsatellite analysis to define loss–of-heterozygosity (LOH), an indication for deleted chromosomal regions. Microsatellites are specific topographic DNA sequences constituted of di- to tetranucleotide repeats for which an individual can be homo- or heterozygous. This allelic homo- or heterozygosity is detected by PCR amplification using primers specific for each microsatellite. After acrylamide gel based electrophoresis, the alleles are detected by autoradiography or fluorescence.

Oligodendrogliomas

In 1994, Reifenberger et al reported association of 1p and 19q chromosomal deletions to oligodendrogliomas (1). Later on, Cairncross associated such 1p-/19q- oligodendrogliomas to a better survival and to a chemoresponse to PVC treatment (2).This was the first report of a predictive criterion for prognosis and therapeutic response in a brain tumour.

Depending on the study, oligodendrogliomas represent 4 to 30% of all reported gliomas (for review 3). This illustrates the lack for reproducible criteria for the differential diagnosis between oligodendroglioma and other gliomas (mainly astrocytoma).

Therefore, we wanted to try to correlate 1p-/19q-oligodendroglioma to histological criteria. For this, we analysed a series of 59 gliomas: 44 reported as oligodendroglioma, 11 as oligo-astrocytoma and 4 as astrocytoma. From 48 of these formaline- fixed and paraffin-embedded gliomas we were able to retrieve DNA suitable for genetic analysis. Microsatellites for chromosomes 1 and 19 as well as for chromosomes 10, 17, 22 which are associated to astrocytoma, were analysed for LOH.

Fig. 1. illustrates the results of two microsatellites for a single tumour (tumoral reference 22442-a). Analysis is always done in duplicate for the tumour and its control (blood). This is to avoid misinterpretation of artefactual monoallelic amplification which sometimes occurs due to weaker quality of the DNA extracted from formaline-fixed tissue.

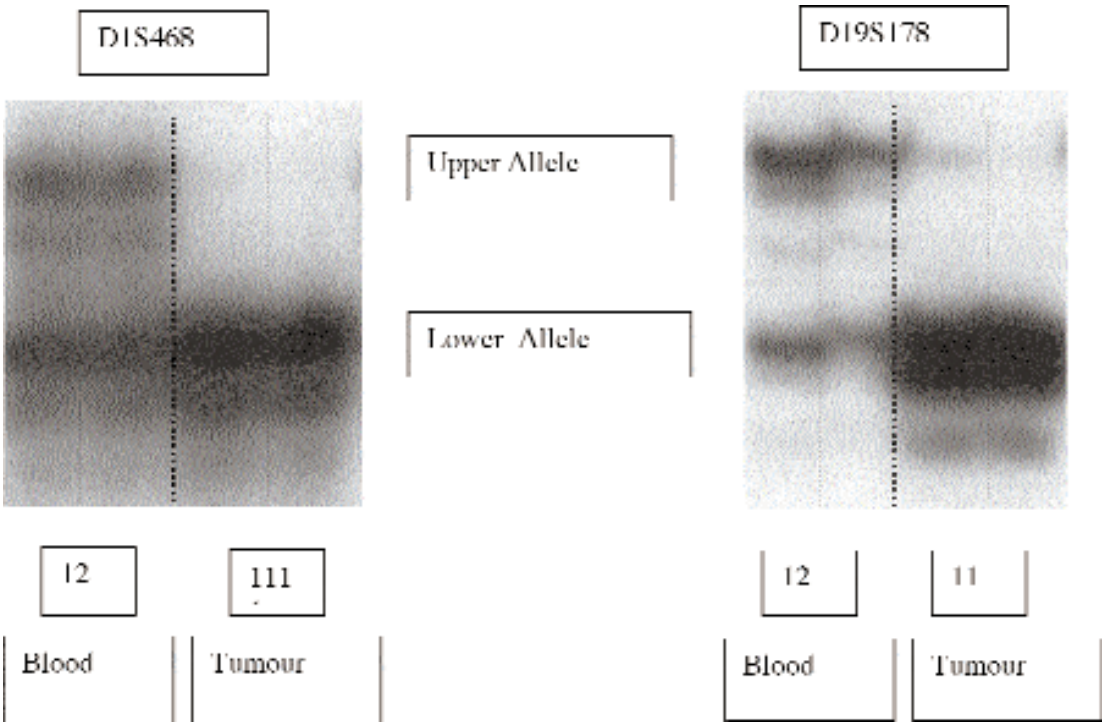

Fig. 1: Autoradiogram of two markers for tumour 22442-a

For both microsatellites, D1S468 and D19S178, two alleles are detected in the patient's blood (score 12 in Fig. 1 and in the table below) while the tumour has only the lower allele (score 11 in Fig. 1 and in the table below). This indicates loss of one allele for both microsatellites in this tumour, and thus of their respective loci on chromosomes 1p and 19q.

The analysis of several consecutive microsatellites on a given chromosome permits to identify the extend of the deleted area. For example, for the tumour (22442-a) in the table below, all analysed microsatellites for 1p and 19q show allelic loss when compared to the patient's blood (CG32)

	22442-a	cg32	
D1S468	11	12	
D1S1612	11	12	
D1S1597	11	11	
GATA29A05	11	12	1p
GATA129H04	11	12	
D1S1728	11	ND	
D1S551	11	12	
D1S1588	11	12	
GATA17G01	11	12	
D19S245	11	12	
D19s112	11	12?	
D19s412	11	12	19q
D19S178	11	12	
D19S246	11	12	
D19S589	11	12	
D19S254	11	12	

Analysing this way these 48 tumours, we identified 22 gliomas with deletions of 1p-/19q. These1p-/19q- gliomas rarely had deletions in the other studied chromosomal regions, chromosomes10, 17 and 22, whereas the non 1p-/19q-gliomas frequently had. This indicates that pathogenesis of gliomas has at least two distinct pathways of tumorogenesis. The low frequencies of common deleted regions observed in the 26 non 1p-/19q- gliomas,did not allow further subclassification of this subgroup of tumours.

To correlate the 1p-/19q- genetic profile to tumour histology, we reviewed the microscopic sections of allstudied gliomas. We found 20 oligodendrogliomas with classical histological features: a very compact, lobulated lesion formed of fried egg-cells or their anaplastic variation (Fig. 2).

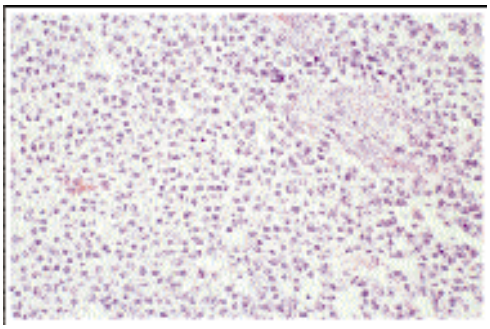

Fig.2: Classical histological appearence of a 1p/19q deleted oligodendroglioma : a compact lesion composed of "fried-egg" tumoral cells

Interestingly, all these 20 classical oligodendrogliomas were 1p-/19q-. The two 1p-/19q- tumours without the classical oligodendroglioma features would be classified as another subtype of oligodendroglioma or oligoastrocytomas, depending on the neuropathologist. Thus, on the basis of this study, 91% of 1p-/19q- oligodendrogliomas can be identified solely on histological criteria (4). This will allow every neuropathologist to identify the chemosensitive oligodendrogliomas even in the absence of genetic analysis. This improved classification of gliomas will allow better evaluation of therapeutic trials .

Ependymomas

Ependymomas are low-grade neuroepithelial tumours accounting for 6-12% of all intracranial neoplasms in children and young adults. The molecular causes are unknown. Cytogenetics has shown some non-specific translocations, as well as variable frequencies of deletions on chromosomes 9q, 10, 13, 17 and 22. Recent CGH (comparative genomic hybridisation) analyses have identified deleted and amplified regions in various chromosomes (for review 3).

We have started a project to define deleted chromosomal regions in a large series of ependymomas. We have collected over 250 formalin-fixed and paraffin-embedded tumours from three different neuropathology laboratories (Brussels, Lille and London) using very strict histological criteria. This series contains ependymomas from different brain regions and from patients of all ages. For some of the tumours, we have also obtained a blood sample to be used as control.

Using 178 tumours of this series for LOH analysis with Weber 8 and 9 set of markers, labelled radioactively or with fluorescence (LICOR DNA analyser), we have so far analysed chromosome 22, as well as parts of two other chromosomes. Over 20 tumours showed large regions of homozygosity for chromosome 22 (Rousseau et al., unpublished). Numerous tumours showed LOH for the two other studied chromosomes (Rousseau et al., unpublished). Our ongoing efforts are aimed at identifying the smallest overlapping deleted regions using additional markers. This will allow the identification of the probable tumour suppresser gene(s) and to associate these deletions to the clinical characteristics.

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

Mechanism of action of reelin.

In 1999, in collaboration with the group of J. Herz (Dallas), it was shown that reelin can indeed physically binds to the extracellular portion of the VLDLR and ApoER2, but not to the closely related LDLR. This study demonstrates that VLDLR and ApoER2 are both specific Reelin receptors (Hiesberger et al, 1999). In 2000, several experiments have been carried out to try and define the part of reelin that is implicated in binding VLDLR and ApoER2. Several partial reelin cDNA constructs have been cloned and expressed in 293T cells to verify that the partial reelin protein is secreted. Some proteins are not secreted and we found that the binding in this case (using the intracellularly trapped protein as the ligand) is inconsistent, presumably because the folding of the protein is not correct. Thus far, or data point to the central part of reelin, particularly repeats 3 to 6, as containing the receptor binding domain. In parallel to binding, we assess the various constructs for their capacity to induce the tyrosine phosphorylation of the Dab1 adapter, as this is the only functional assay for reelin activity. Thus far, most constructs that bind to the receptor induce Dab1 phosphorylation. This study is still largely in progress.

Another question concerns the putative action of reelin on axonal growth. A few studies in the literature showed that reelin deficiency disturb the growth of some axonal bundles, but the question whether this reflects a direct or an indirect effect of reelin on growth cone steering remains unsettled. We addressed this question using the collagen tridimensional assay. Briefly, reelin-deficient (reeler) cortical explants are cultivated in soft collagen to allow axonal outgrowth in absence of reelin. Outgrowing axons are confronted to exogenous sources of reelin such as transfected cells or normal cortical explants, or to control (reeler) explants or cells. Using this system, we showed that the presence of reelin does not influence cortical axonal outgrowth, suggesting that the reported effects of reelin on axons is indirect.

Biochemistry of reelin

Proteolytic processing of Reelin was demonstrated in 1998. Using antibodies directed against the N-terminal region of the protein, three fragments are revealed in western blots corresponding to the full length protein (more than 400 kDa), and two cleavage products of 300 and 180 kDa. Using antibodies against the C-terminal region, three bands of 400, 220-250 and 100 kDa are seen in western. This pattern is compatible with cleavage at two sites. Whereas processing in explant culture in vitro is selectively inhibited by zinc chelators and presumably due to a metalloproteinase, the cleavage pattern described above can also be generated by other enzymes, at least in vitro. This suggests that the processing of Reelin reflects more the folding of the large protein than the activity of a specific processing enzyme. As a consequence, our initial aim of cloning the candidate enzyme implicated in reelin processing was abandoned. The question of the functional meaning of processing remains unsettled and potentially important. Our preliminary binding data show that the part of reelin involved in binding lipoprotein receptors is contained in the middle fragment in between the two cleavage sites. However, full-length reelin also binds to the receptor and it will be necessary to estimate affinities to understand whether the cleavage product binds the receptor with a different affinity.

Using the antireelin antibodies developed previously in our laboratory, we have shown that reelin is present in human CSF and other colleagues have shown that it is detected in plasma, presumably following secretion by the liver. The presence of reelin is CSF raises the question of modifications of reelin is neurological and/or psychiatric diseases; for example, there are a few reports that suggest modifications of reelin expression in psychoses. In order to address such studies, a reliable assay for reelin is required and we have initiated a collaboration with Prof. C. Sindic (UCL, Fac. Médecine) to develop an imunoassay. Thus far, however, classical ELISA sandwich assays do not seem to be sensitive enough and other alternatives are being investigated. Another aspect of this work is the detection of antibodies directed against reelin in certain pathological conditions. This cannot be studied thus far, because the only recombinant reelin available is the mouse reelin. We therefore decided to construct a human reelin cDNA (coding sequence of more than 11 kb) for production of human reelin in transfected cells; this construction is at the final stage.

Cloning of other brain developmental genes and interactions with the reelin pathway

Using two differential techniques, namely representational difference analysis (RDA) and differential display (DD), several genes have been cloned that are candidates for interaction with the reelin pathway. Several candidates correspond to known genes. For example, differential screens identified ROBO, Doublecortin, and (SFRP1) secreted frozzled related protein 1 and a few other genes that are reasonable candidates for interaction of reelin. As these genes are studies actively by other laboratories, we have decided not to investigate them further. In addition, both screens allowed us to clone several unidentified genes, and six of them are currently being studied further. They have features of real genes, expressed in the embryonic mouse brain. For some of them, a message is detected in Northern blot using poly(A) RNA, and in situ hybridation studies are under way to select candidates with an interesting expression pattern. After this further selection, we will focus on the cloning of the full-length cDNA and on the predicted protein.

Analysis of the reelin promoter.

Starting from the partial promoter sequence cloned a few years ago in our laboratory, genomic clones containing the mouse and human reelin promoters have been isolated and the sequence of the promoter region has been extended. Several constuctions using fragments of the reelin promoter to drive a reporter gene have been tested by transient transfection of 293T and P19 cells. These studies have defined a short silencing region located 5' from reelin. Further studies of that region are in progress. Studies of the promoter require in vivo data. Therefore, we have initiated studies using mouse transgenesis experiments. However, mouse transgenesis was not available in our University and we are still in the process of implementing the technology.

Genomic organization of the Dab1 gene

Our laboratory collaborated previously in the demonstration that Dab1 is a key component of the reelin signaling pathway, and cloned and mapped the human DAB1 to chromosome 1p. We also cloned Dab1 orthologs from several amniotes (turtle, lizard and chick). Thus far, the genomic organization of Dab1 genes remains mostly unknown and we have initiated a study to define the complete exon-intron structure of the Dab1 gene in the mouse and in man, to define all splicing forms and to characterize the promoter region. In both species, the Dab1 gene is more complicated that initially thought. Although Dab1 is a relatively small protein, the largest form being 555 amino-acids, the gene is complex and extends over large regions of genomic DNA. Many alternative forms of Dab1 result from alternative splicing. In addition and most interestingly, at least 4 different promoters have been found thus far (using the 5' RACE technique). This suggests that different promoters could be used in different neuronal classes. This study of the Dab1 gene are reaching completion. After they are complete, our attention will shift towards analysis of the different promoters.

Reelin and brain evolution

Our studies of reelin expression during brain development in various amniotes has been pursued with analysis of embryonic chick (Bernier et al., 2000). In addition, a few crododilian embryos were obtained with the collaboration of J-Y. Sire (Paris) and a part of the reelin cDNA sequence was cloned from crocodile RNA. We could demonstrate that our antireelin antibody 142 stains reelin producing cells in the embryonic crocodile brain. We now have all the material to perform a study of reelin protein and mRNA expression during cortical development in that species.

The set of comparative data on reelin expression was used to formulate an hypothesis on the role of the reelin signaling pathway during cortical evolution (Bar et al., 2000). Briefly, our data suggest that the reelin signaling pathway was necessary to allow the evolution of the cortex to proceed from a simple, poorly laminated structure in stem amniotes to the ordered, multilaminar cortex in mammals. An argument for this model was provided by studies from the Walsh lab, who showed that, in man, mutations in reelin generate a lissencephalic phenotype.

Reelin and the development of the human embryonic cortex

Some of our antibodies react well with human reelin and allow the study of reelin-positive cells by immunohistochemistry on paraffin sections. In collaboration with Meyer (Tenerife), the development of reelin-expressing cells in the human fetal cortex has been studied from 11 gestational weeks to birth. At 11 gestational weeks (GW), the neocortical marginal zone contained a single layer of reelin-positive mono-or bipolar horizontal Cajal-Retzius (CR) cells. From 14 GW onward, the subpial granular layer (SGL) invaded the marginal zone, initially containing reelin-negative cells. In parallel to the emergence of the SGL, a second population of reelin-positive cells appeared in the marginal zone. These cells, provisionally named CR-like cells, were intermediate in size between granules of the SGL and CR cells. The density of reelin-positive

cells remained stable from 16 to 24 GW, despite the large tangential growth of the cortical surface, suggesting that the actual number of reelin-positive neurons increased during development. Most CR cells disappeared after 27 GW, in parallel with the dissolution of the SGL. These observations show that different neuronal populations of reelin-expressing neurons are present in the fetal human MZ, and that a possible function of the SGL is to supply reelin-producing cells through a gradual transformation of reelin-negative granules into reelin-positive CR-like cells, thus coping with the protacted neurogenesis and dramatic surface expansion of the human neocortex. These studies have been extended using additional markers and have allowed a better definition of the early development of the human cerebral cortex. (Meyer et al., 2000).

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This work supported by the Foundation is part of a research programme aimed at the study of the neuroendocrine regulation of reproductive function. During this past year, we have pursued two lines of research, i.e. further studies on the metabolism of steroids in rat hypothalamic tissue and further studies on the particularities of the guinea pig model for the study of neuroendocrine regulation of gonadotropin secretion. Furthermore, this year has been a transition year in our programme, during which we concentrated our efforts at establishing in our laboratory new research tools (pituitary and hypothalamic cell lines).

In vitro studies of the hypothalamic "GnRH pulse generator" : regulation and functional organisation of the hypothalamic neurosecretory system responsible for pulsatile secretion of GnRH

Neurosteroid metabolism in hypothalamic tissue

Gonadal steroids play a key role in the regulation of the activity of both the neurosecretory system responsible for the intermittent secretion of GnRH and the pituitary gonadotrophs responsible for gonadotropin secretion, trough negative and positive feedback actions. Presently, we still have an only limited understanding of the role of hypothalamic sex steroid metabolism on GnRH and gonadotrophin secretion. Hypothalamic tissue has the capacity to synthesize and biotransform steroids, mechanisms responsible for the production of so called neurosteroids, which includes both androgen- and progesterone derivatives. In this context, we have been more particularly interested in the regulation of hypothalamic 5α-reductase, 3α-oxydoreductase and 20α-oxydoreductase activity, which are involved in the biotransformation of testosterone and progesterone into potentially bioactive neurosteroids.

The mechanism that controls the enzymatic pattern and activity of these enzymes in the brain is not yet fully elucidated. Some steroids may play an important role in the regulation of 5α-reductase activity, but their effect would depend on the type of tissue. In a previous study of our laboratory, the activities of the different progesterone transforming enzymes in the hypothalamus of the male rat were estimated in vitro. Considerably higher 5α-reductase and 3α-oxydoreductase enzyme activities were found before puberty (rats of 10 to 20 days) than in adulthood (rats of 90 days), resulting in a higher conversion of progesterone into 3α-progesterone and 5α, 3α-progesterone in the prepubertal stage than in the mature rat. We have pursued a series of studies to evaluate the influence on this pattern of activity of adrenalectomy and/or castration performed at an early stage of prepuberty, to study whether the 3α-reduction is affected during inhibition of the 5α-reductase activity, to study the evolution of the hypothalamic enzyme activity during aging. Furthermore, we also initiated a study aimed at examining the endogenous production of 3α-reduced and 5α-reduced progesterone metabolites in the hypothalamus of the rat.

The main results can be summarized as follow :

- age dependency differ to a limited extend somewhat as to the type of enzyme. The 5α-reductase activity is highest in young rats (18 days), decline to about 30% of these values in mature rats (90 days old) and remain at that level thereafter with, however, a tendency for increase in the older rats ($\geq$270 days). The 3α-oxydoreductase activity is lower than the 5α-reductase activity at all ages, but is also highest at prepuberty, to decrease thereafter to levels of about 25% of the initial activity and remain unchanged thereafter up to 365 days.

Castration at day six of life did not markedly reduce the high prepubertal enzyme activities, neither did adrenalectomy combined with castration ; castration, in turn, did not prevent the decrease of enzymatic activity occurring around the age of puberty. From these results, it

can thus be concluded that the high prepubertal values are not explained by the action of sex steroids secreted by the adrenals and that the peripubertal decrease of enzymatic activity is independent of the pubertal increase of serum testosterone.

- results obtained with finasteride, an 5α-reductase inhibitor, illustrate that it is possible to differentially modulate the hypothalamic 3α-OR and 5αR-activity, an inhibition with finasteride resulting in a relative increase of the 3α-OR/5αR-activity ratio with increased production of 3α-progesterone.
- As a next step in our investigations, in order to gain further insight into the potential significance of the age-related changes in hypothalamic progesterone-transforming enzymes, we initiated a study aimed at the estimation of the endogenous hypothalamic content of 3α- and 5α-reduced progesterone metabolites as determined by HPLC followed by GC/MS. In extracts of pooled hypothalamic tissue from both prepubertal and postpubertal rats, measurable amounts were found for 5α-progesterone, 5α,3α-progesterone and 3α-progesterone. In these experiments, no relevant differences could be demonstrated between the hypothalamic content of those metabolites in prepubertal as compared to postpubertal animals. Taken into account that the measured concentrations of these neurosteroids are dependent not only on the relative activity of the different involved enzymes system, but also on the availability of substrate (precursors) that in turn, may be produced partly in situ and be parts blood born, the next step in our investigation will be to assess the endogenous hypothalamic concentrations following manipulation of the endogenous circulating steroid concentrations (adrenalectomy and castration with and without administration of progesterone/testosterone).

Neuroendocrine regulation of gonadotrophin secretion in the male guinea pig model

Using an in vitro model of superfusion of male guinea pig hypothalamic explants, we have described in previous experiments unexpected responses to excitatory amino-acids with, in particular, NMDA-receptor mediated inhibition of gonadotropin-releasing hormone (GnRH) release from hypothalamic explants of intact male guinea pigs. This response is opposite to that previously described for other species, in particular the rat. We observed that this inhibitory response is reversed to a stimulatory effect mediated through NMDA-receptors for hypothalamic explants obtained from long-term orchidectomised guinea pigs. We have subsequently shown that the NMDA-receptor mediated inhibitory response on GnRH secretion for hypothalamic explants of intact males is mediated by opioidergic systems.

Taken the unexpected results of the in vitro experiments concerning GnRH release, we have then embarqued in a systematic study of the luteinizing hormone (LH) response to excitatory amino-acids in vivo. As a first result, these experiments, which have necessitated the set up of a specific bioassay for measurement of guinea pig LH (see below), have shown a stimulatory effect of NMA on LH secretion in both intact and long-term orchidectomized male guinea pigs in vivo. This stimulatory response is prevented by pretreatment with the NMDA-receptor antagonist AP-5. We thus observe a differential response to NMDA-receptor activation for the in

vitro GnRH release by intact male guinea pig hypothalamic explants and the in vivo LH secretion in intact males, with respectively an inhibitory and stimulatory effect. We are now trying to unravel the mechanisms underlying these seemingly opposite responses. In an ongoing series of experiments, we are trying to differentiate in vivo between hypothalamic and direct pituitary effects, trough assessment of the response to NMA and AP-5 with and without pretreatment of the animals with a GnRH-antagonist (cetrorelix). Initial results gave indication that the stimulatory response of LH secretion upon NMA administration may be at least in part not GnRH-mediated. This would suggest two possible working hypothesis, i.e. that NMA exerts direct stimulatory effects on LH secretion at the pituitary level and/or that the LH response following NMA administration is mediated by other hypothalamic neuropeptides (increased secretion into the portal system of neuropeptides with stimulatory action on the pituitary gonadotrophs or decreased secretion of neuropeptides or neuromodulators with inhibitory effects on the gonadotrophs). Before exploring these two working hypothesis, we presently perform experiments to confirm more definitely whether the response of LH to NMA is indeed (partly) independent of hypothalamic GnRH (different regimens of GnRH-antagonist pretreatment ; systematic comparison of the ability of cetrorelix to block the response to NMA and different dosages of guinea pig GnRH). Once these experiments are completed, we will turn our attention to a possible role of opioidergic systems and NPY.

Unique species specific characteristics of the guinea pig neuro-endocrine regulatory system of reproduction

Since we have been working for now over 10 years with the guinea pig models, it became increasingly clear that the guinea pig present some unique features of species specificity and, in fact, there is increasing evidence that the guinea pig may have been taxonomically misplaced with the rodents. These features of species specificity were accompanied by practical methodological problems, with ensuring delay to some parts of our projects, and they complicate the interpretation of results in terms of extrapolation to other species. However, these particularities may also help to gain insights in the neuro-endocrine regulation of reproduction. A first major surprise in recent years has been the fact that guinea pig GnRH has a unique structure with two amino-acid substitutions as compared to the major form of the decapeptide that is found in all other mammalian species studied to date. In in vivo experiments, we have observed that both in the rat and in the guinea pig, mammalian GnRH is a more potent stimulator of LH secretion than guinea pig GnRH. This may be due either to higher affinity of mammalian GnRH for both the rat and the guinea pig GnRH-receptor and/or guinea pig GnRH is more rapidly degraded by proteolytic enzymes in vivo than mammalian GnRH in both the rat and the guinea pig. To further explore these issues, we are pursuing experiments along three lines :

- in vitro studies of the degradation of guinea pig and mammalian GnRH by rat and guinea pig serum ;
- in vitro binding studies of guinea pig and mammalian GnRH to GnRH-receptors in homogenates from rat and guinea pig pituitaries and on an immortalised LH secreting pituitary cell line of murine origine ;
- we are presently initiating efforts aiming at cloning the gen for the guinea pig GnRH-receptor.

At this stage of our experiments, it appears that the difference of response to guinea pig and rat GnRH probably resides in differences in the interaction of the GnRH molecules with the GnRH-receptor molecules, rather than in differences in in vivo degradation of the peptides.

In our in vivo experiments, we have experienced that also LH of the guinea pig displays unusual species specificity. Indeed, the well established in vitro bioassays for measurement of LH with use of testicular Leydig cells from rat or mice testis, successfully applied to measure LH from a variety of mammalian species, were shown to be inadequate for measurement of guinea pig LH, as guinea pig LH fails to stimulate testosterone secretion from Leydig cells from these two species. Therefore, we had to set up a specific bioassay for measurement of guinea pig LH, using dispersed Leydig cells from guinea pig testes.

Establishment of new research tools

In previous years, for our studies of the neuro-endocrine regulation of reproduction, we have used two experimental models : in vitro GnRH secretion by the isolated mediobasal hypothalamus of the male guinea pig (either in a static incubation system or in a continuous superfusion incubation system) and in vivo secretion of LH in the male guinea pig with use of a specific bioassay for measurement of guinea pig LH. In the past year, we have now validated a new experimental model, i.e. the in vitro LH secretion by an immortalised pituitary cell line of murine gonadotrophic origine that acquired the capability to secrete intact LH after permanent transfection with the rat gene for the bèta chain of LH (gift from P. Mellon ; Centre for Molecular Genetics, University of California at San Diego). In the validation of this model we have demonstrated in the cell line the presence of the estrogen-receptor (ERa), we have demonstrate the presence of GnRH-receptor mRNA by RT-PCR, we have demonstrated cellular calcium mobilisation in response to exogenous GnRH by fluorescence technique (pretreatment of the cells with Fluo-3) and, finally, we have demonstrated that we can measure both spontaneous and GnRH-stimulated LH release in the cell-culture medium and modulation of this response by estrogens.

Presently, we are also working on the establishment and validation of cultures of an immortalised GnRH secreting cell line from murine hypothalamic origine.

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Report of the Research Group of

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Study on the functional regulation of the neurotransmission

During year 2000, two different projects have benefit from the financial support of the FMRE :

Characterization of the functional coupling of the neurotensin receptor with muplitple G-proteins

• Evidence for the dual coupling of the rat neurotensin receptor with pertussis toxin-sensitive and insensitive G-proteins. Gailly P., Najimi M. & Hermans E. (2000) FEBS Lett., 483, 109-113.

• Interaction with $G\alpha_i$ and $G\alpha_q$ protein subunits promotes high affinity agonist binding to the neurotensin receptor NTS-1 expressed in E.Coli.' Hermans E. & Grisshammer R. submitted to Biochem. J.

The effects of the neuropeptide neurotensin (NT) are thought to result from its high affinity interaction with specific membrane receptors. Early binding studies conducted on brain tissues and cell lines led many authors to propose the existence of multiple NT binding sites. Three NT receptor subtypes have now been cloned and named NTS1, nts2 and nts3. NTS1 and nts2 belong the large family of G-protein coupled receptors whereas nts3 corresponds to the previously characterized gp95/sortilin containing a single transmembrane domain. In contrast to nts2 and nts3, that were only clearly identified in the late 90ies, NTS1 has received much more attention and was extensively studied in a large variety of cell lines, tissues and recombinant systems for more than a decade. The coupling of this receptor with G-proteins is demonstrated by the ability of guanylyl nucleotides and Na^+ ions to modulate the binding affinity of NT agonists. Although both positive and negative controls of adenylyl cyclase by NT were reported in some cultured cells expressing NTS1, most of the effects of NT have been attributed to the activation of PLC. This suggests the involvement of $G_{q/11}$-type G-proteins and accordingly, many responses to NT are unaffected by the inactivation of $G_{i/o}$-type G-proteins by PTx or *N*-ethylmaleimide. However, this is not the rule and a number of authors have also proposed a possible coupling of NT receptors with other signaling pathways that are proposed to involve PTx-sensitive G-proteins. Using transfected CHO cells expressing NTS1 (CHO-NTR), we previously demonstrated the functional coupling of the rat neurotensin receptor NTS1 with G-proteins on transfected CHO cell homogenates by showing modulation of agonist affinity by guanylyl nucleotides and agonist mediated stimulation of $[^{35}S]$-GTPγS binding. In the present study, we observed that $G_{i/o}$-type G-protein inactivation by pertussis toxin (PTx) resulted in a dramatic reduction of the NT-induced $[^{35}S]$-GTPγS binding whereas the effect of guanylyl nucleotide was almost not affected. As expected, NT-mediated phosphoinositides hydrolysis and intracellular calcium mobilization were not altered after PTx treatment. This suggests the existence of multiple signaling cascades activated by NT. Accordingly, using PTx and the PLC inhibitor U-73122 we showed that both signaling pathways contribute to the NT-mediated production of arachidonic acid. These results support evidence for a dual coupling of the NTS1 with PTx-sensitive and insensitive G-proteins. Simultaneous coupling of membrane receptor with different G-proteins has frequently been reported, especially when using recombinant models in which high levels of receptor expression may facilitate the detection of either promiscuous or specific but low-efficient coupling. A variety of responses induced by NT in vivo were shown to be dependent on

$G\alpha_{i/o}$-proteins and/or inhibited by PTx. However, the nature of the NT receptor subtype(s) expressed in these models was not identified. Moreover, in some of these studies, the responses tested are those frequently proposed not to depend on the activation of NTS1 (analgesia, hypothermia). In contrast, the present study provides a clear-cut evidence for an independent functional coupling of the NTS1 to both PTx-sensitive and PTx-insensitive G-proteins. Such functional coupling of NTS1 with both $G\alpha_q$- and $G\alpha_{i1}$-proteins was observed in a model of *Escherichia coli* expressing NTS1-$G\alpha$ fusion proteins. To analyse the coupling specificity of $G\alpha$ subunits to the rat high-affinity neurotensin receptor NTS-1, fusion proteins were expressed in *Escherichia coli* with various $G\alpha$ subunits covalently linked to the receptor C-terminus. The presence of a $G\alpha_q$ subunit or a chimaeric $G\alpha_{i/q}$ subunit, in which the six C-terminal amino acid residues of $G\alpha_{i1}$ were replaced with the corresponding residues of $G\alpha_q$, increased the percentage of receptors in the agonist high-affinity state. This effect was less pronounced for wild-type $G\alpha_{i1}$ and not observed for a chimaeric $G\alpha_{i/s}$ fusion protein. Functional coupling of neurotensin receptor to $G\alpha$ was demonstrated by neurotensin-induced [^{35}S]GTPγS binding for the chimaeric $G\alpha_{i/q}$ and wild-type $G\alpha_{i1}$ subunits with similar agonist potencies, but not for $G\alpha_{i/s}$. We conclude that neurotensin receptor interacts specifically with both $G\alpha_q$ and $G\alpha_{i1}$ proteins, but not with $G\alpha_s$. Differences in agonist and nucleotide binding behavior suggest preferential coupling of neurotensin receptor with $G\alpha_q$ proteins compared to $G\alpha_{i1}$ proteins, or the existence of distinct receptor contact sites involved in the recognition of these signaling partners.The existence of such multiple coupling is likely to have physiological and pharmacological consequences, leading to rather complex responses to agonists. For instance, it would be of interest to show whether the regulation profile of these distinct signaling pathways differ, as suggested for other G-protein coupled receptors.

Glutamatergic modulation of the neuronal dopamine transporter

• Increased dopamine uptake in striatal synaptosomes after treatment of rats with amantadine. Page G., Peeters M., Maloteaux J.M. & Hermans E. (2000) ' Eur. J. Pharmacol., 403, 75-80.

• Modulation of the neuronal dopamine transporter activity by the metabotropic glutamate receptor mGluR5 in rat striatal synaptosomes through phosphorylation mediated processes. Page G., Peeters M., Najimi M., Maloteaux J.M. & Hermans E. (2001) J. Neurochem., in press (accepted 02 October 2000)

The neuronal dopamine transporter (DAT) is an integral membrane protein primarily involved in the clearance of extracellular dopamine (DA) from the synaptic space (Amara et al., 1993). Such a process of reuptake plays a key role in terminating synaptic transmission and in regulating the concentration of DA available for binding to presynaptic and postsynaptic receptors. The DAT is not only a major site of action for psychostimulants such as cocaine and amphetamine which block the reuptake of released DA but also a mode of entry of neurotoxins which destroy DA neurons, e.g. 6-hydroxyDA. Functional impairment of DA transport obtained by pharmacological inhibition or in DAT knockout animals results in profound physical, physiological and behavioural changes. On the basis of this essential role of the DAT in the modulation of dopaminergic transmission, the study of its regulation is of considerable interest.

Elucidation of the DAT primary sequence through cDNA cloning has revealed the presence of potential phosphorylation sites for protein kinases, including protein kinases A and C (PKA and PKC) and calcium calmodulin dependent kinase II (CaM kinase II). The presence of these sites has raised widespread interest in the possibility that DAT undergoes functional regulation by phosphorylation. However, there is little information available regarding the nature of physiological stimuli that contribute to the endogenous control of the DAT function. Based on the close relationship between glutamatergic and dopaminergic systems in the striatum, we investigated the modulation of the DAT activity by metabotropic or ionotropic glutamate receptors.

Short-term incubations of rat striatal synaptosomes with micromolar concentrations of the group I mGluR selective agonist, *(S)*-3,5-dihydroxyphenylglycine were found to significantly decrease the DAT capacity and efficiency. This alteration was completely prevented by a highly selective mGluR5 antagonist, 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP). The effect of *(S)*-3,5-dihydroxy-phenylglycine was also inhibited by staurosporine and by selective inhibitors of protein kinase C and calcium calmodulin dependent protein kinase II. Co-application of okadaic acid prolonged the transient effect of the agonist, supporting a critical role for phosphorylation in the modulation of the DAT activity by mGluRs. In conclusion, we propose that striatal mGluR5 contribute to the control of the DAT activity through concomitant activation of both protein kinase C and calcium calmodulin dependent protein kinase II. Further experiments will be performed in order to precise whether mGluR5 operate a direct control of the phosphorylation state of the DAT. This acute and functional modulation of the DA transport capacity and efficiency by the mGluR5 is probably involved in the well-documented glutamatergic control of the dopaminergic activity in the striatum. Obviously, such a regulation of the DAT by an endogenous neurotransmitter could have profound effects on the intensity and duration of dopaminergic transmission in neurophysiology and in dopaminergic neurodegeneration.

The second part of this study aimed at investigating the effect of short- and long-term treatments with amantadine on the activity of the neuronal dopamine transporter (DAT) in the rat striatum. For this purpose, the [^{3}H]dopamine uptake was measured in striatal synaptosomes prepared from rats treated for 2, 7 and 14 days with amantadine (40 mg/kg; i.p.). After 7 days of treatment, amantadine increased the apparent V_{max} by 30% without modification of the apparent K_m of dopamine uptake whereas no change in these parameters was observed after 2 and 14 days treatment. Binding assays conducted with [^{3}H]GBR-12935 on membranes prepared from animals treated with amantadine revealed no difference in the density and the affinity of striatal DAT binding sites as compared to control. This indicates that the increased dopamine uptake was not reflecting a modification at the level of the DAT expression. The activity of the DAT is regulated by phosphorylation and one may propose that ionotropic glutamate receptors present on presynaptic terminals directly modulate this phosphorylation. An indirect mechanism would involve presynaptic dopamine receptors that control the activity of the DAT in response to the increased dopamine concentration in the synaptic cleft. This study demonstrated that a long-term treatment with amantadine increases the dopamine transporter activity. This probably reflects a compensatory mechanism to the enhanced release of dopamine in the synaptic cleft and constitutes a relevant example of *in vivo* regulation of the DAT activity by a drug that does not directly interact with dopaminergic systems.

Research on Alzheimer's disease.

During year 2000, three different projects have benefit from the financial support of the FMRE:

A GG nucleotide sequence of the 3' untranslated region of amyloid precursor protein mRNA plays a key role in the regulation of translation and the binding of proteins. Mbella EG, Bertrand S, Huez G, Octave JN. Mol Cell Biol. 20 (2000) 4572-4579.

A gene located on human chromosome 21 encodes the amyloid precursor protein (APP) of Alzheimer's disease. Following transcription, the alternative splicing of the primary transcript leads to 10 different mRNAs encoding 10 APP isoforms. In addition, the alternative polyadenylation of the APP mRNA generates two molecules, the former containing 258 additional nucleotides in the 3'untranslated region (3'UTR). We have shown that these 258 nucleotides increase the translation of APP mRNA injected in *Xenopus oocytes*. This mechanism occurs in CHO cells as well. The 3'UTR containing 8 nucleotides more than the short one allows to recover an efficiency of translation similar to that of the long 3'UTR. Moreover, the two guanine residues located at the 3' end of these 8 nucleotides play a key role in the translational control. Using gel retardation mobility shift assay, we have shown that proteins from *Xenopus oocytes*, CHO cells and human brain specifically bind to the short 3'UTR but not to the long one. The two guanine residues involved in the translational control inhibit this specific binding. These results indicate that there is a correlation between the binding of proteins to the 3' UTR of APP mRNA and the efficiency of mRNA translation, and that a GG motif control both binding of proteins and translation.

The processing and biological function of the human amyloid precursor protein (APP): lessons from different cellular models P. Kienlen-Campard, B. Tasiaux and J.-N. Octave Exp. Geront. 35 (2000) 843-850.

One of the major neuropathological hallmarks of Alzheimer's disease is the presence of senile plaques in vulnerable regions of CNS. These plaques are formed of aggregated amyloid peptide. Amyloid peptide is released by the cleavage of its precursor (APP). The establishment of cell lines expressing human APP allowed to characterize both amyloidogenic and non-amyloidogenic pathways of APP catabolism and to identify some of the proteins involved in this processing (known as secretases). This led to a better comprehension of amyloid peptide production, which needs to be further characterized since γ-secretase is as yet not identified; moreover, we still lack a clear overview of the interactions between APP and other proteins promoting Alzheimer's disease (tau, presinilins...). An important limitation of these cell lines for studying the mechanisms involved in Alzheimer's disease is supported by the observation that human APP expression does not modify transfected cells survival. The infection of primary neuronal cultures with a recombinant adenovirus encoding the full-length human APP indicates that APP expression induces neuronal apoptosis by itself; this neurotoxicity does not rely on extracellular production of APP derivatives (secreted APP, amyloid peptide). It is now essential to understand, in neuronal models, the production, localization and involvement of amyloid peptide in neurodegenerative processes.

The role of presenilin-1 in the gamma-secretase cleavage of the amyloid precursor protein of Alzheimer's disease. Octave JN, Essalmani R, Tasiaux B, Menager J, Czech C, Mercken L. J Biol Chem. 275 (2000) 1525-1528.

The most common causes of familial Alzheimer's disease are mutations in genes encoding presenilins (PS) 1 and 2. These mutations alter APP processing and cause increased production of the high amyloidogenic Aß 42. Moreover, PS1-deficient mice show decreased g-secretase processing of APP. PS are hydrophobic proteins that cross 6-8 times the membrane of the endoplasmic reticulum. A limited portion of PS undergoes endoproteolysis and the resulting N- and C-terminal fragments are localized predominantly in the Golgi. Presenilins are homologous to proteins involved in vesicle transport or in the Notch developmental pathway in the nematode *Caenorhabditis elegans*, and PS1-deficient mice show developmental abnormalities consistent with altered Notch signaling. Signaling through the receptor protein Notch requires ligand-induced cleavage of Notch. The recent demonstration that PS1 deficiency reduces the proteolytic release of the Notch intracellular domain indicates that PS1 regulates both APP processing and Notch signaling by influencing protein cleavage events. Since PS1 is required for both release of intracellular domain of Notch and γ-secretase cleavage of APP, it has been proposed that PS1 acts by facilitating the activity of the protease involved or is the protease itself. Human APP695 and PS1 were coexpressed in Sf9 insect cells, in which endogenous g-secretase activity is not detected. In baculovirus infected Sf9 cells, PS1 undergoes endoproteolysis and interacts with APP. However, PS1 does not cleave APP in Sf9 cells. In CHO cells, endocytosis of APP is required for Aß secretion. Deletion of the cytoplasmic sequence of APP (APPDC) inhibits both APP endocytosis and Aß production. When APPΔC and PS1 are coexpressed in CHO cells, Aß is secreted without endocytosis of APP. Taken together, these results conclusively show that, although PS1 does not cleave APP in Sf9 cells, PS1 allows the secretion of Aß without endocytosis of APP by CHO cells.

Report of the Research Group of

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INTRODUCTION

As usual, this activity report is split into two main parts, supervised respectively by Pierre Maquet and Eric Salmon. The first part pertains to the studies of states of altered consciousness (sleep, coma, vegetative state) and of the neural correlates of implicit and explicit processes in memory. The second one deals with memory functions, gestural praxis and degenerative diseases.

RESEARCH PROGRAM ON ALTERED STATES OF CONSCIOUSNESS

Sleep studies

Our studies were designed to look at experience-related changes in brain activity during human sleep. The basic idea was to train normal subjects to a specific task in the afternoon then scan them, during sleep, in the subsequent night.

The project was conducted by Pierre Maquet, with the contribution of Steven Laureys and Philippe Peigneux. We used the *probabilistic serial reaction time* (PSRT) task described by Axel Cleeremans (Research Associate FNRS, ULB), who was involved in the project. For the details of the task, see Jimenez, L., Mendez, C. & Cleeremans, A. [1996, J. Exp. Psychol.: Learning, Memory and Cognition 22, 948-969]. The study was also run in collaboration with Carlyle Smith (Professor, Dept Psychology, Peterborough, Canada), a leading world specialist on sleep-memory functional relationships.

Three populations were studied. In a control waking population (N=7), the subjects were scanned at rest and while they were performing the PSRT task. In the experimental population (N=6), the subjects were trained to the PRST task then scanned in the post-training sleep. In the control sleep population (N=5), subjects were not trained to the task then scanned during sleep.

Shortly, the analysis consisted in finding the [group (sleep trained vs sleep non trained) by condition (REM sleep – wakefulness)] interaction then finding the conjunction between this set of areas and the areas activated by the execution of the task in group 1. Hence this analysis was intended to evidence the brain areas active during PSRT practice at wake and re-activated during the subsequent REM sleep.

Indeed, our results showed that some of the brain areas needed for the execution of the task are more active in REM sleep in trained than in non trained subjects. These results thus show a experience-dependent re-activation of cerebral regions during post-training REM sleep. As subjects improved their performance the day after, we surmise that these reactivation might be related to some processing of memory traces during REM sleep. Further studies will be needed to confirm this latter hypothesis.

These data are now published in Nature Neuroscience.

The left premotor cortex was among the re-activated areas. Further analyses showed that this brain structure was more correlated with the posterior parietal cortex and the supplementary area in trained than in non trained subjects during REM sleep. This outstanding finding means that brain areas are not reactivated in isolation but participate to neural networks, possibly optimised during post-training sleep (see figure 1).

The manuscript gathering these results is submitted.

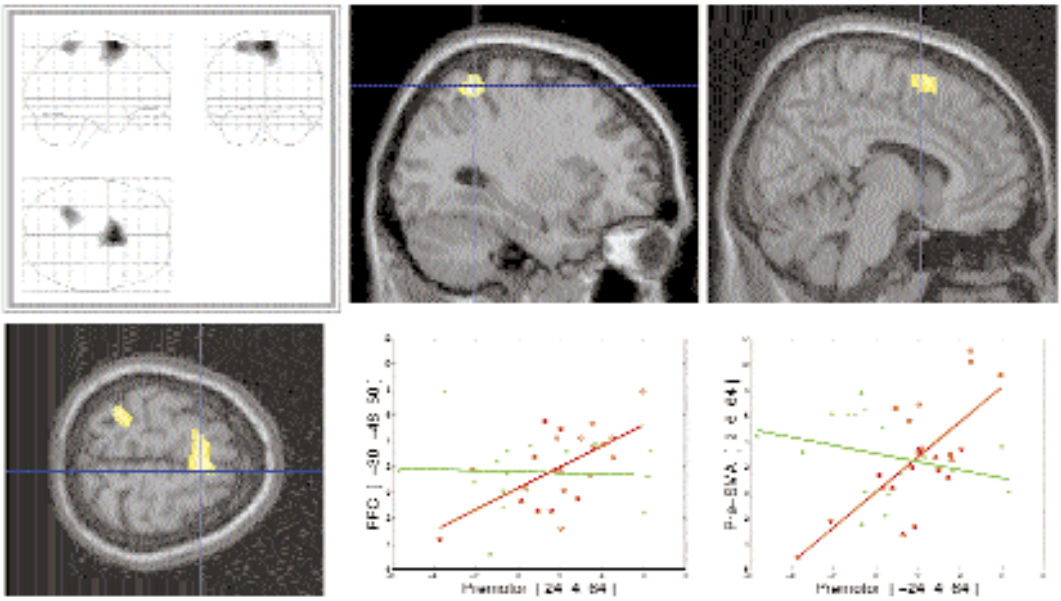

Figure 1. Significant group (trained versus non trained) by left premotor rCBF interactions in the pre-supplementary motor area (pre-SMA) and posterior parietal cortex (PPC). SPM{T} is displayed in glass brains [top left] within the standard reference frame and thresholded at $P < 0.001$ (uncorrected). Significant loci of activations in the PPC [top middle, bottom left] and pre-SMA [top right, bottom left] are shown superimposed on an individual T1-weighted MRI brain template. Plots of the adjusted and centred rCBF of [bottom middle] the left premotor cortex (abscissa) and PPC (ordinate) and of [bottom right] the left premotor cortex (abscissa) and pre-SMA (ordinate). For both, the functional relationships between these 2 areas are significantly different in trained subjects (red) than in non trained subjects (green).

Another analysis of our data provided evidence that the ocular saccades are differently generated in REM sleep than during wakefulness. During REM sleep, ocular movements are significantly correlated with regional cerebral blood flow in the occipital cortex and the geniculate body, which was not the case during wakefulness. From these results, we hypothesise that ocular movements during human REM sleep are triggered by mechanisms similar to the Pontine-Geniculo-Occipital (PGO) waves in animals.
The manuscript concerning these results is submitted.

Future directions

Sleep studies are now going on, using subjects trained to the same task, except that the sequence of the stimuli is random and not generated by an artificial grammar. These new subjects would thus be submitted to the same visuo-motor learning (reaction time task) but not to the rules of the artificial grammar. Contrasting the two groups of subjects might provide some evidence that the networks subtending the learning of the complex material embodied in the grammar are also reactivated during REM sleep.

Moreover, increasing the number of subjects trained to a serial reaction time task and the number of subjects scanned during sleep without previous training would allow us to run a random effect analysis. The latter would allow us to confirm the published results and extend our inferences to the general population.

The acquisitions for this study should be finished by Spring 2001.

Another sleep study is already scheduled, now exploring the experience-dependent reactivations during human sleep following an explicit memory task. This task is the exploration of a virtual maze. It capitalises on what we know at the cellular level on cellular reactivation during post-training sleep in rats. It is also using a task successfully developed by Eleanor McGuire at the FIL (WDCN, UCL, London) who collaborated on this project as an expert of functional neuroimaging in human spatial memory.

This study should begin by spring 2001.

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Study of patients with altered states of consciousness

This project was run by Steven Laureys and Pierre Maquet, in collaboration with 2 Anaesthesiologists (Dr Marie-Elisabeth Faymonville and Dr. Nathalie Janssens). This is a prospective study of cerebral glucose metabolism at rest and cerebral blood flow at rest and under *auditory or somato-sensory stimulation* in vegetative state patients.

Vegetative state is characterised by preserved arousal but abolished awareness of self and environment. Our results confirm that, in vegetative patients, the cerebral glucose metabolism is decreased by 40 to 60 % as compared to normal subjects. We were the first to map brain areas that are systematically most impaired in VS. These areas were localised in polymodal associative cortices (bilateral prefrontal regions, Broca's area, parieto-temporal and posterior parietal areas and precuneus) (published in NeuroImage and cover of Physiological Imaging of the Brain using PET) and were shown to be functionally disconnected from both thalami (presumed intralaminar nuclei) (Book Chapter in Physiological Imaging of the Brain using PET). Interestingly, recovery of awareness of self and environment was paralleled by a functional recovery of metabolism in these same cortical regions (results published in Lancet as a Correspondence Letter). Moreover, we were able to show that the altered cortico-thalamo-cortical modulation in vegetative state, regained near normal values after recovery of awareness (results published in Lancet as a Research Letter).

We than showed that external (auditory or noxious somatosensory) stimulation is still able to activate the primary cortices but not the associative cortices. Furthermore, in vegetative patients, functional connectivity is impaired along the usual processing streams of these inputs. The data concerning auditory stimulation are now published in Brain. A manuscript is in preparation for the somato-sensory data. It sheds light on the clinically very relevant issue of pain perception and suffering in patients in the vegetative state. In summary, even though the patients' metabolism of the brain was much lower than normal (less than half of normal values), some regions still showed significant activation during noxious stimulation: the brainstem, the thalamus and the primary somatosensory cortex. However, a large network of hierarchically 'higher-order' multi-modal association areas failed to activate: the secondary somatosensory cortices, the insular regions, the posterior parietal and prefrontal areas and the anterior cingulate cortex (regions that are known to be involved in pain affect, attention and memory). Moreover, primary somatosensory cortex, the only cortical region that activated in vegetative patients, was no longer functionally connected with the rest of the brain (i.e., the 'higher order' brain regions thought to be necessary for conscious processing) (see fig. 2). Our results show that severely depressed brain regions in patients in a vegetative state can be activated by peripheral noxious stimuli. This activation, however, remains limited to subcortical and primary cortical regions. As the observed cortical activation is isolated and functionally disconnected it can-

not lead to the integrative processes that are thought to be necessary for the attainment of the normal level of awareness. Interestingly, in those patients who subsequently recovered consciousness, the brain returned to near normal functioning and restored long-range functional connectivity several months after the brain insult.

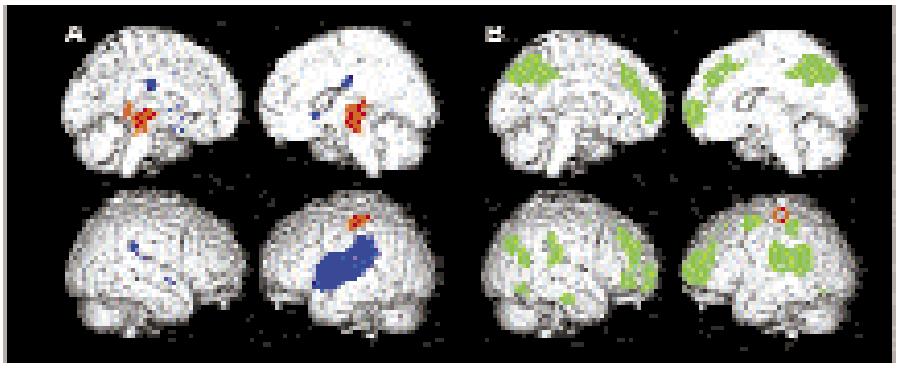

Figure 2: A. Brain areas showing an increase in blood flow (rCBF) in vegetative state patients during noxious somatosensory stimulation that are common to controls , shown in red, and areas where rCBF showed significantly less activation compared with controls, shown in blue ($P<0.001$). B. Brain areas that showed a significant difference in modulation with primary somatosensory cortex (red circle [-42 –26 58]) in vegetative patients compared with controls, shown in green.

At the patient's bedside, it is very difficult to evaluate residual cognitive function in patients in the vegetative state. This is even more so in the minimally conscious state, a recently defined clinical condition (American Congress of Rehabilitation Medicine, 1995) characterised by preserved visual fixation and emotional or motor behaviour that is contingent upon the presence of specific eliciting stimuli (e.g., family voices). Using PET we were able to show that **minimally conscious patients** show a very different pattern of brain metabolism compared to the clinically closely related vegetative state patients (figure3, unpublished results).

The locked-in syndrome is a tragic condition characterised by preserved consciousness and nearly complete absence of voluntary motor output. Classically, vertical eye movement and eyelid blinking are intact and provide the exclusive mechanism for communication with the outside world. Again, assessment of cognitive function is difficult because voluntary movements may be very small, inconsistent and easily exhausted. We recently demonstrated that while there is no single cortical voxel that shows a significant decrease in resting metabolism, an increased activity is observed in bilateral mesiotemporal areas, encompassing the amygdalae. Previous PET studies in normal volunteers have demonstrated specific amygdala activation in relation to fear (Morris et al., Nature, 1996;383:812-5). Even if it is difficult to make judgements about locked-in patient's thoughts and feelings, the increased activity in the amygdalae correlates with the terrifying situation of an intact awareness of self and environment in a sensitive being, locked in an immobile body (fig. 4) (this paper is in preparation).

These data pertain directly to a wider field of research in cognitive neuroscience: the neural correlates of consciousness. As shown in figure 3, widening our research to patients variously conscious, we can approach this issue.

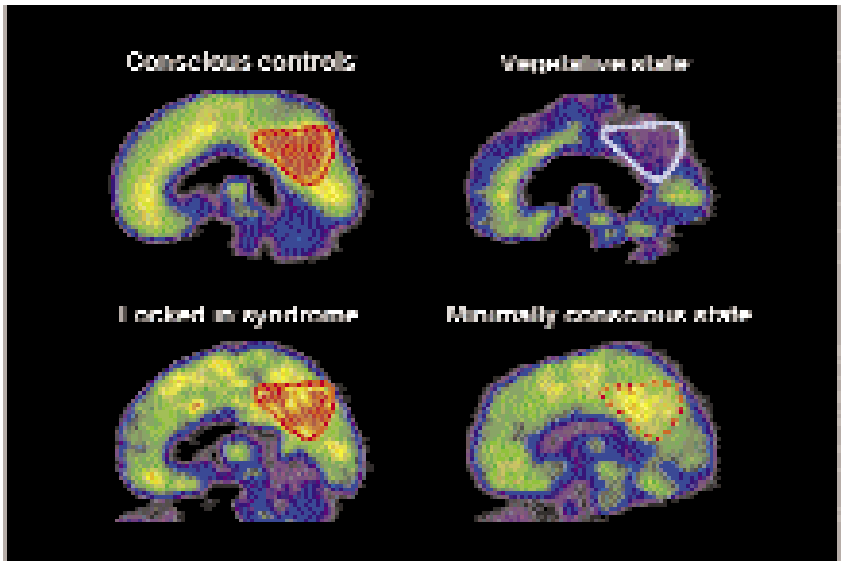

Figure 3: Averaged, spatially normalised images of resting cerebral metabolic rates for glucose shown in a saggital plane and represented according to the same colour scale. Note that in normal healthy controls (n=35), the medial parietal area (precuneus) and posterior cingulate cortex (delineated by a red line) is the metabolically most active region of the brain; in the vegetative state (n=19), this same area (delineated by a dashed blue line) is most impaired cortical region; in the locked-in syndrome (n=1) the activity in this areas is preserved; and in the minimally conscious state (n=3) the activity is intermediate but clearly different from that observed in vegetative state.

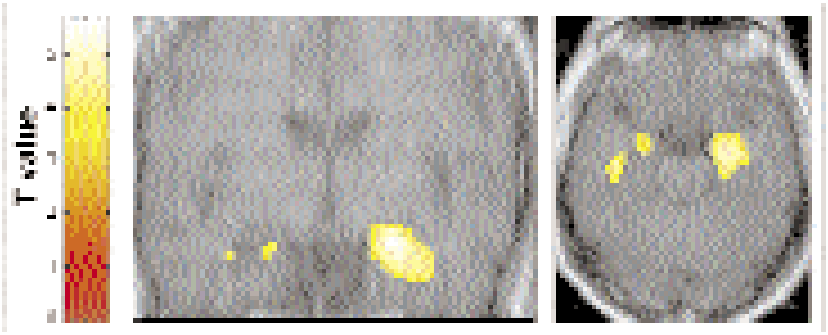

Figure 4: Regions that showed significantly increased metabolism in the patient with a locked-in syndrome compared to 21 controls, projected on the patient's coregistered and normalized MRI ($p<0.001$).

Future directions

The study of patients with altered states of consciousness is now expanding its recruitment area and becoming truly multi-centric. In Liège (CRC), patients are referred from the university hospital (CHU) and from many different peripheral hospital and rehabilitation centres. In Brussels (PET Unit, Erasme Hospital – Collaboration with Dr. Serge Goldman), patients are referred from the Hospital itself, from the academic hospital AZ-VUB Jette (collaboration with Prof. Guy Ebinger) and from nearby rehabilitation centres (Petits Abeilles, CTR – Brugmann). Activation studies using 15O labelled water are planned in Erasme Hospital in February 2001. The same activation paradigm will be used in both PET centres (CRC and Erasme), exploring brain responses to visual (static and virtual motion) and emotionally loaded auditory stimuli in patients with altered states of consciousness compared to healthy controls.

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- Positron emission tomography in coma and vegetative state. French Society of Clinical Neurophysiology, Symposium on Coma, Brain Death and Vegetative State. December 12-13, 2000, Paris, France.
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RESEARCH PROGRAM ON IMPLICIT AND EXPLICIT MEMORY PROCESSES

Study of neural correlates of implicit learning

The Cleeremans's task alluded above was central to the sleep project. In consequence, we also explored in great detail the neural correlates of the implicit acquisition of the probabilistic rules which govern the succession of the stimuli in the practised sequence. This project was run by Philippe Peigneux and Pierre Maquet on waking subjects. Since a wide interindividual variability was observed in the acquisition of the higher-order contingencies of the sequential material, we designed a new procedure in order to account specifically for the between- and within-subject behavioral variance in the analysis of PET data. Using this approach, we were able to show that the striatum is more activated when behavioral data demonstrate successful use of the probabilistic rules of the grammar, than when behavioral data did not evidence any learning of these probabilistic contingencies (Figure 2). Additional frontal activation was also found, which confirm and extent previous researches showing that the learning of the higher-order material included in the sequence during a SRT task involves a fronto-striatal network.

These results are published in Human Brain Mapping.

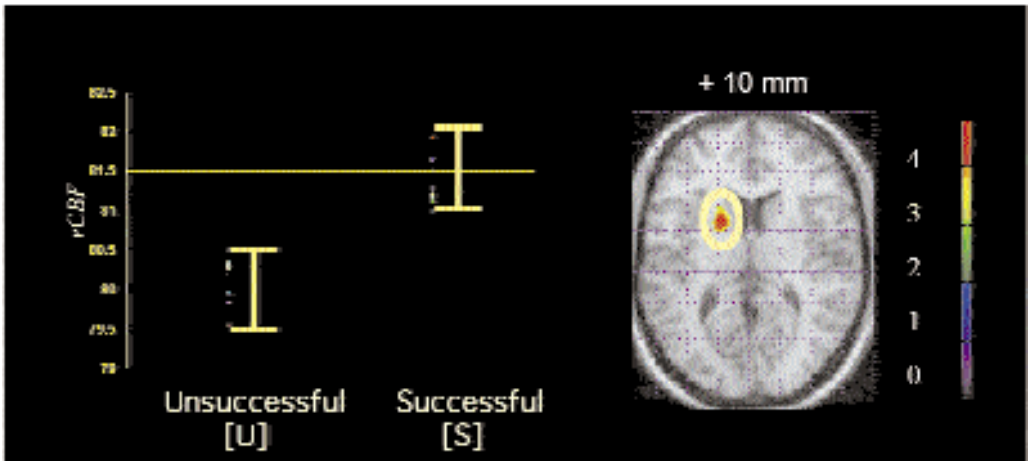

Figure 2. Significant activation at the voxel-level (pcorr. < .05) in the anterior striatum; coordinates -16 8 10 mm in the standard stereotaxic atlas of Talairach et Tournoux (1988). Left panel show rCBF response pattern at this voxel in [S]uccesful (i.e., the sequence is implicitly learned) or [U]nsucessful (i.e., no consistent learning) conditions. Horizontal bars indicate minimal and maximal observed rCBF values across all participants.

Study of neural correlates of conscious sequence generation

Building on the collaboration initiated for the sleep studies, we run a protocol proposed by Axel Cleeremans and his fellow Arnaud Destrebecqz (now Research Assistant FNRS, ULB). The basic idea was to use the *process dissociation procedure* (PDP) proposed by Jacoby (J. Mem. Lang., 1991, 30, 513) in a sequence learning paradigm. This PET study was part of the doctoral thesis of Arnaud Destrebecqz.

Before scanning, subjects practised a deterministic SRT task, in which a second-order 12-elements sequence was presented continuously during a 20 minute learning session. Afterwards and during PET scanning, subjects were instructed that a sequence was repeated continuously during the task, and that they will have now to generate sequences in two conditions. In the inclusion condition (3 scans), subjects were asked to generate the sequence practised during the learning session. They were instructed that if they cannot remember explicitly the sequence, they must try to guess it. In this inclusion condition, the performance is supposed to rely on both implicit and explicit processes. Rather in the exclusion condition, subjects were asked to generate any sequence that is completely different from the one they think they have learned. In this exclusion condition, continued generation of all or part of the learned sequence is thought to reflect the influence of implicit processes only, because explicit resources are devoted to avoid reproducing the sequence.

Twenty-one subjects were scanned in the exclusion and inclusion conditions, 17 subjects remain for the statistical analysis after examination of their behaviour during the practice of the SRT task. An interaction analysis between the performance score (i.e., the number of generated triplets elements whose succession belongs to the learned sequence) and the generation condition (inclusion versus exclusion) intended to disclose the brain areas specifically involved in the explicit contribution to the generation performance. Statistical parametric mapping results showed an activation of the fronto-polar – pregenual anterior cingulate area and of the dorsomedial thalamus. We suggest that these areas are part of a brain network specifically involved in the conscious retrieval of sequential information.

The manuscript gathering these data is in preparation. Preliminary results were presented at the Human Brain Mapping meeting in San Antonio (USA) and at the Meeting of the Association for the Scientific Study of Consciousness (ASSC) in Brussels.

After having focused on the neural correlates of conscious processing of sequential information, an ongoing study aim to investigate the neural substrates specifically involved in the implicit – non conscious - processing of sequential information during the generation task. A study conducted by Destrebecqz and Cleeremans (Pyschonomic Bulletin, in press) showed that the level of consciousness of the sequence can be manipulated depending on the response – stimulus (RSI) interval. In our first study, RSI was 250 msec, and most of the knowledge was explicit. Destrebecqz and Cleeremans data showed that when RSI is 0 msec, most of the knowledge is rather implicit and little is consciously available. Hence we run a new study with a RSI set to 0 msec. Comparisons between the two studies will allow to delineate better the neural circuitry involved in the implicit processing and retrieval of sequential information.

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RESEARCH PROGRAM ON MEMORY FUNCTIONS, GESTUAL PRAXIS AND DEGENERATIVE DISEASES

Alzheimer's disease, memory and executive functions

The starting point of this program is the recent demonstration of early executive dysfunction in Alzheimer's disease (AD), concomitantly with episodic memory troubles (Delbeuck, 2000). In the literature, executive dysfunction is frequently associated with frontal impairment, but the pathophysiology might correspond to intracerebral dysconnexion, noteworthy between frontal and posterior cortices. Early occurrence of executive dysfunction in AD is in contrast with predominant posterior cortical involvement observed with functional imaging, showing major decrease of activity in multimodal integrative regions such as posterior cingulate (Salmon, 2000). When contrasting neuropsychological performances in population with and without significant frontal hypometabolism, there are no significant differences for tasks tapping executive functions (Collette, abstract 2000). This suggest a predominant role for anterior-posterior functional dysconnexion.

On the other hand, inhibition functions might be more specifically related to frontal activity. In the Hayling paradigm, one must inhibit an automated response consisting in giving the last word of a very familiar sentence (for example: god save the …), to produce an answer without any relationship with the beginning of the sentence. Performance at that task is related to the metabolic level in prefrontal areas (Collette, 2000). Based on that preliminary result, we designed a study in healthy volunteers, showing that conceptual inhibition is related to activation in several frontal areas (submitted).

Another ongoing experiment will explore brain regions involved in executive functions such as integration of informations compared to simultaneous concurrent processing of information.

Among several executive functions attributed to the central executive of working memory, one is the possibility to associate processing from different cognitive systems, such as long term and short term memory. We did not observe frontal activation for such an interaction between systems. When contrasting serial recall of words versus non-words (that have not any representation in long term memory), we essentially demonstrated lexico-semantic temporal and parietal activation. So long term representations of words contribute to an improvement of performances in short term memory (submitted). Subsequent studies are planned to explore relationships between working memory and language.

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Corticobasal degeneration, motor slowness and upper limb apraxia

The starting point of this project is our interest for corticobasal degeneration (CBD), a degenerative disease characterised by asymmetrical movement disorder and, reputedly, upper limb apraxia. First, we emphasised the network of brain regions that are metabolically impaired in CBD using the resting [18]fluorodeoxyglucose technique with PET, comparing their brain images with those of patients suffering from Parkinson's disease or progressive supranuclear palsy. Our results showed that those hypometabolic cerebral areas in CBD predominantly consist in perirolandic, premotor and anterior parietal cortex, and thalamus.

The manuscripts summarising these results are now published (Movement Disorders; Journal of Neurology)

When measuring motor slowness during repetitive movements (finger tapping or hand pronosupination), one can observe important variability between patients. A contrast of brain metabolism between slow and very slow patients showed that supplementary motor area is functionally impaired in the later group (Garraux et al., abstract 2000). Moreover, new programs for data analysis allow to directly compare functional and anatomical data, and preliminary results show that cerebral atrophy is not sufficient to explain SMA hypometabolism in CBD (Figure 1; Garraux et al., abstract 2000).

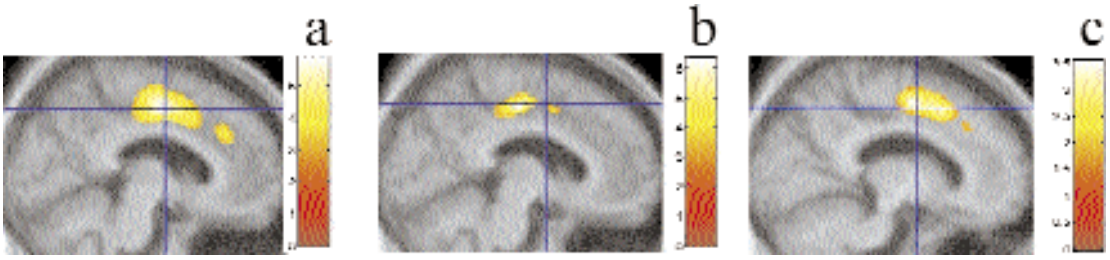

Figure 1. Brain areas related to motor slowness in CBD and projected on a parasagittal section of the mean normalized T1-weighted MRI from the 10 CBD patients. a) : comparison of 18FDG-PET data; b) comparison of MRI data of grey matter (MRIGM); c) comparison between 18FDG-PET and MRIGM data. For display, results were thresholded at $P < 0.001$, uncorrected excepted in the comparison between PET and MRIGM data ($P < 0.05$, uncorrected). The vertical line corresponds to $y = 0$ while the horizontal line corresponds to $z = +50$ mm in the Talairach space (Talairach and Tournoux, 1988)

These data are part of the doctoral thesis (in preparation) of Gaëtan Garraux.

Apraxia is reputedly a main symptom in CBD, but the characteristics of apraxia in CBD and its neural substrates were rarely studied extensively. In a large population with CBD, we administered an apraxia testing battery based on recent cognitive models [Peigneux et Van der Linden (2000), Revue de Neuropsychologie, 10(2), 311-362]. Two different scores for apraxia were used, which reflects complementary cognitive contributions to the gestural impairment. A cut-off score for apraxia was defined with regard to each score, and the regional cerebral metabolism of CBD patients with apraxia was compared to that of patients without apraxia. We showed that CBD patients have a higher overall frequency of errors than controls, and that the

metabolic level in the anterior cingulate gyrus differentiates CBD patients with and without apraxia. At variance, when the measure was the ability to correct an error in a second trial, we showed that CBD patients are unable to correct their errors at the same rate than control subjects did for imitation modalities only, demonstrating the presence of visuo-imitative apraxia in CBD. Moreover, metabolic activity in a fronto-parietal network differentiates CBD patients with and without apraxia using this latter score.

Visuo-imitative apraxia was also investigated in a single-case study, where we disclosed in addition a significant deficit to imitate gestures which necessitate to access to body knowledge representations. PET demonstrated bilateral hypometabolism in the medial dorsal pathway was thought to induce the difficulties to integrate the visual information (provided by the demonstration of the gesture to imitate) with regard to the body knowledge, resulting in a gesture production deficit during imitation specifically

The manuscript gathering these results are published or submitted for publication (Peigneux et al., 2000; Peigneux et al., submitted).

To further challenge theoretical models of apraxia that describe the different stages for mimicking movements, we first contrasted visual analysis of gesture and that of objects in normal volunteers, using oxygen 15-labelled water in a PET activation study. We showed that the bilateral lateral temporo-occipital regions (BA 19/37) and the motion-related area MT/V5 are specifically involved in the first stages of the visual analysis of gestures, before access to their "lexical" content (see Figure 2)

The manuscript gathering these results is published (Peigneux, 2000). These later studies were part of the doctoral thesis of Philippe Peigneux.

We have concentrated in a further activation PET study on the other processing stages involved in gesture reception and production. Eighteen volunteers were asked to imitate meaningless and meaningful gestures, to pantomime to command, or to perform an action semantic task. The analysis of these data is still in progress, but first evidences indicate that gestures representations are stored in brain regions very close, but inferior, to the brain areas involved in the visual analysis of gestures (Peigneux et al., abstract 2001). Distribution of the cerebral activation profiles according to the experimental conditions partly supports the organisation of gestural information processing proposed in recent cognitive models of apraxia.

The analysis of these data is in progress. A manuscript will be prepared in the first semester 2001.

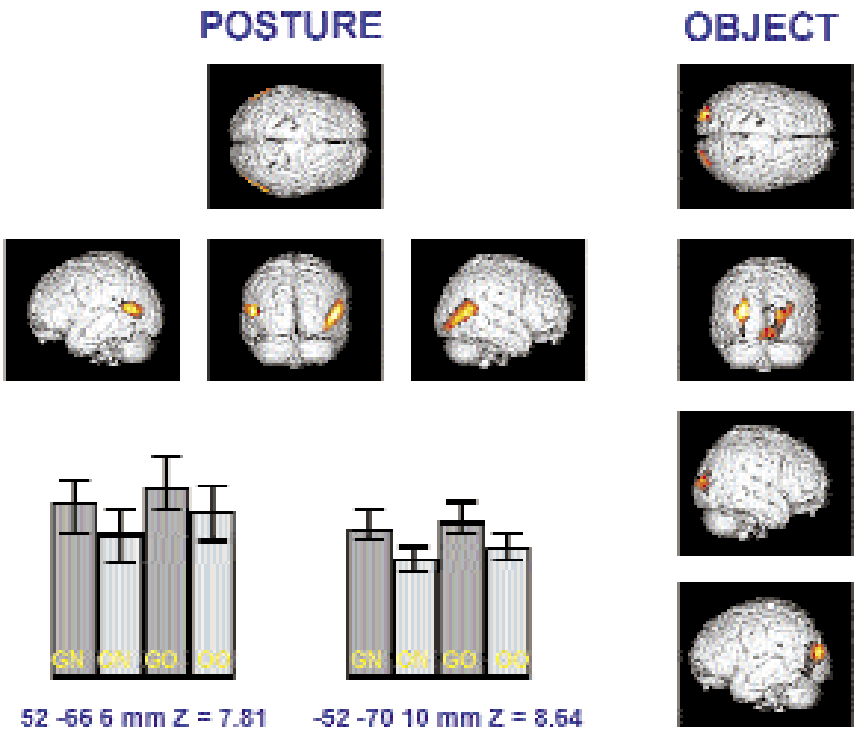

Figure 2. Brain Areas Disclosed During Posture and Object Visual Processing. Brain areas where rCBF was significantly associated with visual processing of intransitive upper-limb postures (left panel side) and intransitive objects (right panel side) during conjunction analysis. Rendered on a template MRI image at the level of peak activation (voxel-level corrected for multiple comparisons, p corr. < 0.05) in top, rear, and lateral (right and left) perspective. The mean rCBF is illustrated for each experimental condition in the two highest significant voxels of the lateral occipito-temporal junction, activated in posture-related conditions, one in each hemisphere. Mean rCBF is displayed with minimal and maximal rCBF values measured across all subjects. Condition abbreviations : [PN] Meaningful posture naming; [PD] Meaningless posture orientation decision; [ON] Meaningful object naming; [OD] Meaningless object orientation decision.

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Report of the Research Group of

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Interim Report

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

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Neuregulin 1 (Nrg1) isoforms have been shown to influence the emergence and growth of oligodendrocytes, the CNS myelin-forming cells. We have investigated how Nrg1 signaling of ErbB receptors specifically controls the early stages of oligodendrocyte generation from multipotential neural precursors (NP). We show here that embryonic striatal NP synthesize NRG-1 transcripts and proteins, as well as ErbB2 and ErbB4 -but not ErbB3 receptors. Striatal 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.

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

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Cultured cortical oligodendrocyte progenitor cells (OPCs) express the ionotropic glycine receptor (GlyR) and glycine transporters 1 and 2 (GLYT1, GLYT2) which, when activated, allow an intracellular calcium influx secondary to a depolarization-induced opening of voltage-gated calcium channels (VGCC). Considering this effect on intracellular calcium concentration in OPCs, we hypothetized that glycine could alter potentially calcium-dependent metabolic events such as proliferation and differentiation. OPCs proliferation was quantified by a bromodeoxyuridine incorporation assay and differentiation by measuring the relative proportions of

A2B5, O4 and galactocerebroside immunophenotypes during *in vitro* oligodendrocyte maturation.We show that glycine induces a specific dose-dependent mitogenic effect on A2B5-positive OPCs which is suppressed by nifedipine, a L-type VGCC blocker. Although not completely inhibiting this process at any stage, glycine also significantly decreases the rate of oligodendrocyte differentiation. Without interfering with the *in vitro* survival of oligodendroglial cells, this work supports our hypothesis that glycine can regulate oligodendrocyte development by a mechanism involving a modulation of intracellular calcium homeostasis. Thus, glycine released by neurons, as other neurotransmitters, might serve as a physiological signal between neurons and OPCs during oligodendrogliogenesis. Pharmacological manipulations of this system might also provide new pathways for remyelination strategies.

Since cyclin-dependent kinases (Cdks) and their endogenous inhibitors (CKIs) play an essential role as regulators of cell cycle withdrawal and onset of differentiation within oligodendroglial cells, we assessed here the effects of exogenous chemical CKIs on the proliferation , differentiation and survival of cultured rat cortical oligodendrocyte progenitor cells (OPCs). We demonstrated here that purine derivatives and especially roscovitine strongly inhibit PDGF-induced OPCs proliferation. Roscovitine alone did not affect oligodendrocyte maturation wheras in the presence of mitogenic signals and thyroid hormone, roscovitine increases the generation of fully mature oligodendrocyte probably by triggering the appearance of cell cycle arrested-OPCs that are then available to undergo the committing influence of thyroid hormone. Finally, we provided evidence that antimitotic concentrations of roscovitine prevent from oligodendroglial apoptosis induced by growth factor deprivation while concentrations much higher than the IC_{50} value of its antiproliferative effect enhance apoptosis in PDGF-stimulated OPCs. We thus here demonstrate by *in vitro* experiments that small molecular weight chemical CKIs, which are about being tested in clinical trials for their chemotherapeutic properties, have important effects on oligodendroglial development. This opens prospects for the potential use of these well tolerated agents in remyelination strategies.

Study of developmental Neuronal Migration

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Study of Radialin as an IFAP.

We have investigated the distribution of Radialin, the radial glia protein recognized by the RC2 antibody in mouse embryos and have found that it is present outside of the CNS in forming skeletal muscles (Leprince et al., 1999b). Radialin in muscle cells is also a 300 kDa protein that, unlike the Radialin form in radial glia, is a soluble protein, allowing its characterization and immunoprecipitation. We have compared the properties of Radialin with those of other large MW IFAPS known to be expressed by glial cells during development (Leprince et al., 2000). Of these, IFAP-300, Transitin, Plectin and Synemin differ from Radialin in either their distribution, time of expression or immunoreactivity. Only Nestin, an intermediate filament pro-

tein present in neural precursor cells, has properties that are very similar to those of Radialin, suggesting that the two proteins are highly related, differing possibly only by post-translational modifications or slight antigenic determinants. Some of those data constitute a publication that appeared in Developmental Dynamics (Chanas-Sacre et al., 2000c).

Phenotypic plasticity of the radial glial cells in vitro.

We had shown previously that the morphology of cerebellar glial cells in vitro and their expression of radialin was modified by the removal of serum and in particular of two of its components, thrombin and lysophosphatidic acid (Chanas-Sacre et al., 2000a;Leprince et al., 1999a). Those two agents, known to modulate astroglial cells phenotype, also control in vitro the expression of the 300 kDa protein recognized by the RC2 antibody in cerebellar glial cells. We have further shown that lysophosphatidic acid which preserve the RC2 staining is inhibited by a specific inhibitor (NASPA) and is sufficient to mimic completely the effects of serum. Those results are now incorporated in a paper in preparation (Chanas-Sacré et al.).

Radial glial cells as glial precursors and/or neural stem cells

The origin and regulation of the radial glia phenotype has been discussed in a review paper that we have published in the Journal of Neuroscience Research (Chanas-Sacre et al., 2000b). In this paper we discussed the possibility that the radial glial cells, in addition to their known role of guides for the migrating neurons, are also the precursor cells for mammalian astrocytes and might even be one transient form of neural stem cells. To support this last hypothesis we have started a characterization of the expression of radial glial markers by multipotential stem cells grown in vitro as neurospheres. Indeed we found that most cells in the neurospheres express radialin before starting to express neuronal, oligodendroglial or astroglial markers (Nguyen et al., 2000). Thus, as shown previously for Nestin in neural stem cells, the expression of radialin is a marker of cell immaturity.

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Cell biology for prevention and treatment of sensori-neural deafness

Malgrange, B.[1], Lefebvre, P.P.[1], Belachew, S.[1], Rigo, J.M.[1], Nguyen, L.[1], Van de Water, T.R.[2], Thiry, M.[3] and Moonen, G.[1]

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The organ of Corti is a complex structure containing an invariant mosaic of hair cells (HCs) and supporting cells (SCs). The signals regulating the production if HCs and SCs are actually not known. During development in mice, the production of these cells is completed by E16 and the capacity to produce new cells following an injury is limited to this neonatal period. In order to understand the mechanism underlying this production of new hair cells in the cochlea, we have studied the production of supernumerary hair cells in late embryonic and early postnatal rat organ of Corti. We have shown that supernumerary HCs are produced in the neonatal organ of Corti in culture in response to an incubation in the presence of transforming growth factor Ǝ (TGFƎ) or epidermal growth factor (EGF). This neoformation of sensory cells does not occur through a cell proliferation because of the lack of inhibition by cytosine arabinoside (arac 10^{-5}M) of supernumerary cell production. Conversely, when E19 rat organs of Corti are cultured for 5 days in the presence of arac supernumerary HCs and SCs are observed. One possible mechanism underlying this supernumerary cell production is the inhibition of cyclin-dependent kinases (cdks) particularly cdk 1, 2 and 5, which will induced HCs and SCs differentiation. In order to test this hypothesis, we used specific cdk inhibitors (cdki). Roscovitine (10 :M) and olomoucine (30 :M), two specific cdk1, 2and 5 inhibitors, induced the production of supernumerary HCs and SCs. This phenomenon is dose-dependent and reach a maximum for E19 rat organ of Corti in the developmental study that we have carried out. In the presence of brdU (10-5M) for 18 hours, no proliferative cell was observed in the sensory cell area,demonstrating that the HCs and SCs production is independant from an inhibition of cell proliferation. Another theoretical mechanism which can account for the production of supernumerary hair cell is the induction or the inhibition of apoptotic cell death. When organs of Corti are cultured either in control condition or in the presence of cdk inhibitors, no apoptotic nuclei is seen. The supernumerary HCs and SCs should arise from a direct differentiation of a cell progenitor. This hypothesis is reinforced by previous findings on other cell types showing that roscovitine has the ability to induce cell differentiation through cdk2 inhibition (Lee et al., 1999).

In a parallel investigation, we tried to identify HC progenitors. Analysis of semithin sections has revealed that the supernumerary HCs are produced at the outer edge of the organ of Corti. Ultrastructural studies show that the tectal cells present a morphology which is intermediate between that of Hensen's cell and the HCs, and that Hensen's cells are very similar to Deiters' cells. Quantitative analysis demonstrates that, when the number of HCs increases in the organ of Corti, 1) the total number of HCs + Deiters' cells + tectal cells + undertectal cells + Hensen's cells remains constant, 2) the number of Deiters' cells increases, 3) the number of tectal cells decreases, and 4) the number of Hensen's cells decreases. Immunolabeling of organ of Corti's explants using antibodies specifically directed against a protein expressed early in the

developing hair cell (i.e. Jagged-2) showed that Hensen's cells expressed this early marker when supernumerary hair cell production occurs. These results demonstrate that Hensen's cells retain the capacity to differentiate either into tectal cells which themselves differentiate into HCs or into undertectal cells which are able to differentiate into Deiters'cells.

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Functional significance of neuroreceptors on neural stem cells

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During brain ontogenesis, the temporal and spatial generation of the 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 microenvironnement of neural cells, even at the earliest stages of brain development. It is now established that specific neurotransmitter receptors are present on progenitor cells of the developing central nervous system (CNS) and could play, during neural development, a role that has remained unsuspected until recently (for a review, see publication of Nguyen et al., 2000).

In a recent study using immunocytochemistry and RT-PCR, which will be presented for publication in NeuroReport, we show that glycine ionotropic receptors (GlyRs) are expressed by postnatal rat *striatum*-derived nestin positive cells within neurospheres. By means of whole-cell patch-clamp technique, we have moreover recorded strychnine-sensitive reversible inward currents triggered by glycine in these cells. Our results support therefore the functional expression of GlyRs in neurospheres by nestin positive neural progenitors likely including neural stem cells (NSCs). Furthermore, we have observed that NSCs also show modifications of their membrane conductance in response to the application of other transmitters (GABA, glutamate, acetylcholine and serotonin).

In parallel, we have developed a new culture assay allowing both fast isolation and expansion of rat striatal multipotent progenitors that express PSA-NCAM, a marker which is a hallmark of cells close to NSCs but likely committed to the glial lineage. This defined model of an almost pure cell population will help us to better address the effects of neurotransmitters on neural progenitors proliferation, differentiation and migration capacities.

Astroglial control of neuronal excitability and survival

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As a reminder, the INItoxins are inhibitors of inhibitory neurotransmission (such as NAF or β-carbolines) that are also neurotoxic (NAF is a neurotoxic astroglia-derived factor that also behaves as a negative allosteric modulator of ligand-gated chloride channels).

Interactions of the INItoxins with ligand-gated chloride channels (the GABAAR and the GlyR)

In a previous report, we showed that ∃β-carbolines (βCs), besides their well established negative allosteric modulation of $GABA_A$R-mediated responses, also act on strychnine-sensitive

glycine-gated chloride channels. We had even defined the molecular determinants of βCs-induced inhibition of GlyR function, showing that the inclusion of a β subunit in the channel renders it insensitive to βCs. As 1) the GlyR is also present in the telencephalon where it could even participate in neurotransmission and, hence, 2) could be implied in neuronal excitability disorders, such as epilepsy, and 3) because βCs are proconvulsivant molecules the mechanism of which is not yet fully understood, we looked for an interaction of a panel of anti-epileptic drugs (AEDs) with βCs-inhibition of $GABA_AR$- and GlyR-mediated responses. Among the tested AEDs, levetiracetam was the most efficient and the most potent antagonist of βCs-inhibition on most anionotropic receptors. Clonazepam, pentobarbital and valproate ("GABA active" AEDs) also afforded partial protection while carbamazepine, phenytoin and ethosuximide were uneffective.

Mechanism(s) of βCs-induced neuronal apoptosis

We had previously shown that βCs induce, as do NAF, the apoptotic death of cerebellar granule neurons in primary cultures. Involvement of the $GABA_AR$ in that process had also been suggested as GABA modulators - GABA itself and benzodiazepines - protected neurons against βCs toxicity.

Here we show that this βCs-induced apoptosis i) is neuron-specific as hippocampal or spinal cord neurons are also killed by βCs while astrocytes or fibroblasts remain insensitive, and ii) is not related to an excitotoxic-type of insult since excitotoxins antagonists do not suppress βCs neurotoxicity. To address the mechanism of action of βCs-induced apoptosis, two types of experiments have been performed. Binding/uptake studies using [^{3}H]βCCB on living cells in cultures revealed the existence of two different compartments/sites for [^{3}H]βCCB: 1) a rapidly exchanging one (probably membrane-associated and found on both neurons and astrocytes), and 2) a slowly exchanging one, which most likely corresponds to an intracellular compartment, is selectively neuronal and is enhanced by depolarization. Both sites/compartments could be blocked by GABA, Ro 15-1788 and glycine. This suggests a specific route of entry of βCs into the cell. The mitochondrion is a likely intracellular target of βCs since, upon βCs treatment of neuronal cultures, cytochrome C is released into the cytosol as measured by Western blot,. Finally, as Ro 5-4864 reproduces most of the effects of βCs and as βCs interact with both the low and the high affinity Ro 5-4864 binding sites, we suggest that the peripheral benzodiazepine receptor found in mitochondria could be the molecular target of βCs.

Spinal Cord Traumatic Lesion

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The great diversity of the spinal cord lesions observed in human beings does not find his correspondence in the experimental models intended to study them. Some models, such as medullary section, kinetic compressions with the traditionnal technique of the released weight or the photochemical lesions aimed to study particular aspects of the lesion and regeneration of the injured spinal cord, are imitating only one particular aspect of the human situation. That's the reason why we described first another model of acute compression of spinal cord, who uses a inflatable small balloon introduced into space under-dural in the adult rat and which, contrary to the models classically used, allows to induce a medullary contusion in a closed space. The modulation of the physical parameters of compression (volume of inflation, duration of compression) can modify in a reproducible way the importance of the medullary contusion and can be correlated with behavioral deteriorations which result from it. The detailed study of the evolution of the lesion during the first four weeks following the lesion enabled us to show the massive but transitory regeneration of axons originating from telencephalic regions in the injured CNS. It is associated with the invasion of the lesion by a heterogeneous cellular population made up of macrophages, endothelial cells and especially

Schwann cells which are organized in a screen directed in the large axis of the spinal cord.

Moreover, we developed various techniques of repair of the damaged spinal cord. In particular, we used various cellular types for grafting with the aim to modify the injured site and to bring elements favorable to its rebuilding. As we showed, there is an important axonal regeneration in the injured CNS but it is only transitory and disappear gradually. In our approach of transplantation, we systematically used cells coming from adult syngenic animals in order to place us from the clinical point of view for autograft Schwann cells constitute a particularly interesting cellular stock since they sustain a effective axonal regenetion in the PNS. In the spinal cord lesion induced in our model using the inflatable small balloon, the grafted Schwann cells are quickly integrated. They limit post-traumatic cavitation and the gliosis. They favor a massive axonal regrowth, but the immunohistochimic characterization of fibres demonstrate a local origin starting from the adjacent spinal ganglia with the lesion rather than a telencephalic source. By allowing the axonal growth within the injured CNS, the grafted Schwann cells "peripheralize" the injured spinal cord.

In order to favor the fast elimination of the myelin debris which accumulate in the lesion, we carried out transplantations of peritoneal macrophages. They are able to be integrated in the spinal cord and to reduce the cystic cavitation and do not induce an important gliosis. To a lesser degree than Schwann cells, they are able to support the axonal regrowth coming from spinal ganglia. If they contribute, by reducing the myelin debris accumulated in the lesion, " to decentralize " the CNS, they support also the migration of Schwann cells from the adjacent dorsal roots and by ths way, stimulate te axonal regrowth from spinal ganglia.

Whereas the fibroblasts are often regarded as cells not very useful for the repair of the injured CNS, we showed that the grafted meningeal fibroblasts do not constitute a barrier with pushes back the axons. On the contrary, they are integrated in the injured spinal cord, without causing impenetrable conjunctive scar, limit cystic cavitation and the gliosis. They even support axonal regrowth both from spinal ganglia and from upper-spinal structures such as the downward serotoninergic pathway.

If these various cellular types can bring to the injured CNS an environment sustaining an axonal regrowth, this one is often held without precise orientation. With the aim of supporting the orientation of this regrowth, we used a polymeric matrix directed into the longitudinal direction. This support made up of polylactide is biocompatible and biodegradable. It is integrated into the injured spinal cord and does not prevent a fast cellular invasion including macrophages, endothelial cells and Schwann cells. Moreover, it stimulates

massive axonal regrowth, and the three-dimensional orientation of fibers being primarily longitudinal coming from the adjacent segments with the lesion or even of upper-spinal origin. In addition to the possibility of bringing factors sustaining the axonal regrowth by various cellular types, the acellular matrix allows directional regrowth.

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Brain activations in a dot pattern categorization task.

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Categorization of dot-patterns (Posner and Keele, J. Exp. Psychol., 77, 353, 1968) has been widely used to study mechanisms of natural categorization. To determine the brain regions involved in this sort of categorization, we compared brain activation, using PET, in 2 tasks employing 9-dot patterns. In the categorization task, subjects (n = 14) categorized distorted, novel exemplars of 2 categories and random patterns (3 response choices). The control task consisted of a discrimination of pattern position. Each task was run at 2, matched, levels of difficulty, manipulated by the distortion level or position shift size. Yes/no feedback was provided. Fixation of the fixation target served as baseline condition. The categorization task differentially activated ($z>4.30$; SPM96) the lateral orbitofrontal cortex (BA 11) and 2 dorsolateral prefrontal regions (BA 46 and 9). These regions were not activated in the position discrimination task (vs. baseline condition). Also, the categorization task activated a parietal focus (dorsal intraparietal sulcus) and the cerebellum. The position discrimination task activated also the latter regions, but less so than during categorization. Subtracting categorization from position discrimination yielded a middle temporal (BA 20/21) and medial frontal gyrus (BA10) focus. Both regions showed deactivations in the categorization task (vs. baseline). Task difficulty had no effect. These results show that the dot-pattern categorization task involves a fronto-parietal cortical network. They support a role of orbitofrontal cortex in cognitive tasks that require novel stimulus-response-reward associations.

A dissociation between ventral and dorsal visual streams reflecting the nature of the cognitive task: A pet study.

W.Fias, P.Dupont, B.Reynvoet, S.E.Raigue, L.Mortelmans, G.A.Orban

The aim of the present PET study was to investigate the extent to which processing along the dorsal and ventral streams depends on the cognitive operations required by the task. Two gratings were presented in succession in central vision to subjects (n=14) who responded by pressing a right or left key. In a quantification task, participants had to indicate whether or not the difference in orientations between the two gratings was larger than a fixed standard. In a same-different task, participants had to indicate whether or not the orientations of the two gratings were identical. Detection of dimming of the fixation point was used as a control task. Visual input, motor responses and performance were equated across tasks. Subtracting same-different from quantification yielded significant activation in the right inferior parietal lobule and left ventral precentral gyrus. The reverse subtraction yielded activation in the right occipito-temporal sulcus, confirming earlier results (Orban et al. Eur J. Neurosci. 97, Cornette et al. Soc. Neurosci. Abstr. 98). This pattern of results shows that nature of the task, rather than stimulus attributes or action requirements determines the stream along which information is processed. If the task requires identification for comparison (same-different task) the ventral stream is loaded. Conversely, quantification for comparison requires resources from the dorsal stream.

Cerebral regions processing first and second order motion: A pet study.

P.Dupont, G.Sary, H. Peuskens, G.A.Orban

It remains a controversy whether there are two distinct mechanisms in the human brain for processing first and second order motion. In this study, we used two sorts of stimuli: first and second order gratings which move either to the left or to the right. Subjects (n=8) either discriminated the direction or detected the dimming of the fixation point. Saliency was equated by adding static noise. Discrimination of direction compared to the dimming detection control activated occipital, parietal and premotor regions. The occipital regions were more differentially active for the two types of motion. Direction discrimination of second order motion significantly activated R hMT/V5+ and L and R TRIPS, the junction of the intraparietal sulcus (IPS) and the transverse occipital sulcus. Discrimination of first order motion only significantly activated R TRIPS. Weak activations were observed for the two types of motion in L hMT/V5+ and in R ventral IPS. Finally, there was a weak lingual activation for first order motion discrimination. Most interactions between tasks and type of motion occurred in R hMT/V5+, R STS and L TRIPS, which corresponded to larger activation by the discrimination of second order than of first order motion. Only one region, anterior collateral sulcus, displayed an interaction in the opposite direction. These results are in aggreement with patient studies. In conclusion: when subjects perform a discrimination task with the moving stimuli more occipital regions are active for a second order stimulus than for a first order stimulus.

Separate Neural Correlates for the Mnemonic Components of Successive Discrimination and Working Memory Tasks.

L. Cornette, P. Dupont, G. Bormans, L. Mortelmans and G.A. Orban

We have used positron emission tomography to map the mnemonic components of two tasks at the extremes of the visual short-term/ working memory spectrum. The successive discrimination task requires only storage of a single item for very short time (ultra-short- term memory), while the 2back task requires both maintenance (i.e. storage and rehearsal) and manipulation of several items (working memory). We tested whether or not the storage component, common to the two tasks, engaged the same cerebral regions. To remove unnecessary confounds, we reduced the cues available to the subjects to a single elementary attribute, the orientation of a grating presented in central vision. This prevented subjects from using verbal strategies or vestibular cues and allowed equating of difficulty among tasks. Ultra-short-term memory for orientation engaged a large expanse of occipito-temporal cortex with a rate-dependent antero-posterior gradient: a fast trial rate engaged posterior regions, a slow trial rate anterior regions. On the other hand, working memory for orientation involved the left inferior parietal cortex, left dorsolateral prefrontal cortex and a left superior frontal sulcus region, and to a lesser degree the symmetrical right superior frontal region and a left superior parietal region. Direct comparison of the two orientation memory networks confirmed their functional segregation. We conclude that at least the storage of orientation information engages distinct regions depending on whether or not short-term memory/working memory involves rehearsal and/or manipulative processes.

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A Scientific activities

1. Overview.

Most of the activities of the group has been centered onto G protein-coupled receptors. This family is the largest among membrane receptors. About 150 G protein-coupled receptor types and subtypes have been functionally characterized to date in mammalian species, and a similar number of 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 constituted part of our activities. Finally, a new area of research has been initiated over the past year in the laboratory, which is the study of the ephrin and ephrin receptor families in the development of the central nervous system. This area, which is expected to expand in the future, is described as well.

2. Cloning and expression of orphan neuropeptide receptors

With the aim of identifying the natural ligands of orphan receptors, we have expanded the collection of cell lines expressing genes encoding putative neuropeptide receptors collected in the databases and the human genome sequencing program. Altogether, over fifty orphan receptors have now been cloned, most of them of human origin. The coding region was inserted in a bicistronic expression vector and the recombinant plasmids were expressed in CHO cells adapted to functional screening. The screening is based on the coexpression of a receptor, mitochondrial apoaequorin and $G_{\alpha 16}$, a transduction protein allowing to couple most receptors to the activation of phospholipase Cβ and calcium mobilization. In this system, the cells are incubated with coelenterazine, the aequorin co-factor, for reconstitution of the active form of the enzyme. When the cells are exposed to an agonist of the receptor, intracellular calcium release results in the activation of aequorin, that oxidizes coelenterazine and yields apoaequorin, coelenteramide, CO_2 and light. Light emission is recorded in a microplate luminometer. This assay is used routinely for both the screening of biological activities in complex mixtures and the pharmacological characterization of receptors.

3. Testing orphan receptors for potential agonists.

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

An orphan GPCR, previously designated OR143, was found to be a functional high affinity receptor for Neuropeptide FF (NPFF) and related peptides. NPFF and neuropeptide AF (NPAF) are involved in pain modulation and opioid tolerance. These peptides were known to act through so far uncharacterised G protein-coupled receptors (GPCR). Binding experiments were performed with a new radioiodinated probe, [^{125}I]-EYF, derived from the EFW-NPSF sequence of the rat NPFF precursor. Chinese hamster ovary (CHO) cell membranes expressing

NPFFR bound [^{125}I]-EYF with a K_D of 0.06 nM. Various NPFF analogs and related peptides inhibited [^{125}I]-EYW-NPSF specific binding with the following rank order (K_i): human NPAF (0.22 nM), SQA-NPFF (0.29 nM), NPFF (0.30 nM), 1DMe (0.31 nM), EYW-NPSF (0.32 nM), QFW-NPSF (0.35 nM), 3D (1.12 nM), Met-enk-RF-NH2 (3.25 nM), FMRF-NH2 (10.5 nM) and NPSF (12.1 nM). This pharmacological profile was similar to that reported for the natural receptor expressed in the brain of mice or rats. The stimulatory activity of the same set of peptides was measured by the aequorin assay. The rank order of potency was consistent with the results of the binding assay. Membranes from NPFFR expressing CHO cells bound GTPγ[^{35}S] in the presence of SQA-NPFF (EC_{50} = 19.6 nM). This functional response was prevented by pertussis toxin treatment, demonstrating the involvement of G_i family members. SQA-NPFF inhibited forskolin induced cAMP accumulation in recombinant CHO cells in a dose dependent manner. This response was abolished as well by pertussis toxin pre-treatment (Kotani *et al.* submitted). The cloning of this NPFF receptor was reported by other groups during completion of this work (Elshourbagy et al., J. Biol. Chem. 275: 25965, 2000).

For two other orphan receptors, a number of agonists were identified in a library of random synthetic peptides 12 amino acids long. As these peptides were tested to start with at high concentrations (over 1 micromolar) and unpurified, we next determined a dose response curve for each peptide, and selected the most active peptides for each receptor for resynthesis and HPLC purification. Unexpectedly, these active peptides were found to display high potencies, with EC_{50}'s of 8 nM and 35 nM for the best peptide active on each of the two receptors (Kotani et al. and Laurent et al. unpublished data). We have now synthesised series of deletion mutants and Ala-scans, in order to determine the residues necessary for the biological activity in each case. These peptides are presently being studied. These data will be further used to generate radioligands allowing to perform binding studies. We will also continue to generate peptide variants, in order to improve their affinity. These peptides will allow to study the function of the orphan receptors in vivo.

Orexins are novel peptides identified as the ligands of previously orphan receptors. These peptides are involved in the regulation of feeding behavior. We have studied the signaling properties of the orexin 1 receptor (OX_1), in collaboration with a Swedish group. Ca^{2+} elevations in Chinese hamster ovary cells stably expressing OX_1 receptors were measured using fluorescent Ca^{2+} indicators fura-2 and fluo-3. Stimulation with orexin-A led to pronounced Ca^{2+} elevations with an EC_{50} around 1 nm. When the extracellular [Ca^{2+}] was reduced to a submicromolar concentration, the EC_{50} was increased 100-fold. The inositol 1,4,5-trisphosphate production was also shown to be dependent upon extracellular Ca^{2+}. Fura-2 experiments with the "Mn^{2+}-quench technique" indicated a direct activation of a cation influx pathway, and depolarization of the cells to +60 mV, which almost nullifies the driving force for Ca^{2+} entry, abolished the Ca^{2+} response to low concentrations of orexin-A. The results thus suggest that OX_1 receptor activation leads to two responses, (i) a Ca^{2+} influx and (ii) a direct stimulation of phospholipase C, and that these two responses converge at the level of phospholipase C where the former markedly enhances the potency of the latter (Lund et al. 2000).

4. Purinergic receptors.

Purinergic receptors constitute a subfamily of G protein-coupled receptors that contains 5 functional receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$), and three of these receptors have been described in our laboratory ($P2Y_4$, $P2Y_6$ and $P2Y_{11}$). During the last year, we have examined the expression of mRNA for several P2Y nucleotide receptors by northern blot analysis in purified type 1 cerebellar astrocyte cultures. These results suggest that different P2Y subtypes could be responsible for ATP metabotropic calcium responses in these cells. To identify these subtypes we have studied the pharmacological profile of ATP calcium responses using fura-2 microfluorimetry. All tested astrocytes responded to ATP and UTP stimulations evoking similar calcium transients. Most astrocytes also responded to 2-methylthioATP and ADP challenges. The agonist potency order was 2-methylthioATP > ADP > ATP = UTP. Cross-desensitization experiments carried out with ATP, UTP, and 2-methylthioATP showed that 2-methylthioATP and UTP interact with different receptors, $P2Y_1$ and $P2Y_2$ or $P2Y_4$. In a subpopulation of type 1 astrocytes, ATP prestimulation did not block UTP responses, and UDP elicited clear intracellular Ca^{2+} concentration responses at very low concentrations. 2-MethylthioATP and UTP calcium responses exhibited different sensitivity to pertussis toxin and different inhibition patterns in response to P2 antagonists. The $P2Y_1$-specific antagonist N:(6)-methyl-2'-deoxyadenosine 3', 5'-bisphosphate (MRS 2179) specifically blocked the 2-methylthio-ATP responses. We can conclude that all single astrocytes coexpressed at least two types of P2Y metabotropic receptors: $P2Y_1$ and either $P2Y_2$ or $P2Y_4$ receptors. Moreover, 30-40% of astrocytes also coexpressed specific pyrimidine receptors (Jimenez et al. 2000).

5. Chemokine receptors.

Chemokines are a superfamily of small signaling proteins that play major roles in the recruitment of white blood cells toward the sites of inflammation. They play fundamental roles in the physiology of acute and chronic inflammatory processes as well as in the pathological dysregulations of these processes.

We have also continued to analyze the structure-function relationships of CCR5 and its chemokine ligands. The reported structures of many CC chemokines show a conserved dimer interface along their N-terminal region, raising the possibility that their quaternary arrangement might influence their function. We have analyzed mutants of MIP-1β (generated by Patty LiWang, College Station, Texas, USA) having a range of dimer K_d values in order to determine the significance of dimerization in receptor binding and cellular activation. Functional relevance was determined by receptor binding affinity and the ability to invoke intracellular calcium release from CHO cells transfected with the MIP-1β receptor CCR5. The monomeric N-terminally truncated mutant MIP(9) was able to bind the CCR5 receptor with a K_i of 600 pM but displayed weak agonistic properties, while the monomeric mutant P8A still retained the ability to tightly bind (K_i = 480 pM) and to activate (EC_{50} = 12 nM) the receptor. These data suggest that the MIP-1β dimer is not required for CCR5 binding or activation. In addition, we identified Phe13, the residue immediately following the conserved CC motif in MIP-1β, as a key determinant for binding to CCR5. Replacement of Phe13 by Tyr, Leu, Lys, and Ala showed the aromatic side chain to be important for both binding to CCR5 and chemokine dimerization (Laurence et al. 2000).

CCR5 is the major coreceptor for macrophage-tropic strains of the human immunodeficiency virus type I (HIV-1). Homozygotes for a 32 bp deletion in the coding sequence of the receptor (CCR5D32) were found to be highly resistant to viral infection, and CCR5 became therefore one of the paradigms illustrating the influence of genetic variability onto individual susceptibility to infectious and other diseases. We investigated the functional consequences of 16 other natural CCR5 mutations described in various human populations. We found that 10 of these variants are efficiently expressed at the cell surface, bind [^{125}I]-MIP-1β with affinities similar to wtCCR5, respond functionally to chemokines, and act as HIV-1 coreceptors. In addition to Δ32, six mutations were characterised by major alterations in their functional response to chemokines, as a consequence of intracellular trapping and poor expression at the cell surface (C101X, FS299), general or specific alteration of ligand binding affinities (C20S, C178R, A29S), or relative inability to mediate receptor activation (L55Q). A29S displayed an unusual pharmacological profile, binding and responding to MCP-2 similarly to wtCCR5, but exhibiting severely impaired binding and functional responses to MIP-1α, MIP-1β and RANTES. In addition to Δ32, only C101X was totally unable to mediate entry of HIV-1. The fact that non-functional CCR5 alleles are relatively frequent in various human populations reinforces the hypothesis of a selective pressure favouring these alleles (Blanpain et al. 2000).

The frequency of the Δ32 allele of the CCR5 gene in the population of Northern Africa was determined in collaboration with a French group (Elharti et al. 2000).

A novel ligand of CCR5 was also identified in the frame of our strategy to identify natural agonists of orphan receptors. Hemofiltrate CC chemokine (HCC)-1 is a recently described human chemokine that is constitutively expressed in numerous tissues and is present at high concentrations in normal plasma. Using a cell line expressing CC chemokine receptor (CCR)5 as a bioassay, we isolated from human hemofiltrate an HCC-1 variant lacking the first eight amino acids. HCC-1[9-74] was a potent agonist of CCR1, CCR3, and CCR5 and promoted calcium flux and chemotaxis of T lymphoblasts, monocytes, and eosinophils. It also blocked entry of HIV-1 strains using CCR5 as coreceptor. Limited tryptic digestion of HCC-1 generated the active variant. Conditioned media from several tumor cell lines activated HCC-1 with a high efficiency, and this activity could be inhibited by serine protease inhibitors. Our results indicate that HCC-1 represents a nonfunctional precursor that can be rapidly converted to the active chemokine by proteolytic processing. This process represents an additional mechanism by which tumor cells might generate chemoattractant molecules and recruit inflammatory cells. It might also affect HIV-1 replication in infected individuals and play an important role in AIDS pathogenesis (Detheux et al. 2000).

6. Characterization of a mouse knock-out model for the A_{2a} adenosine receptor.

Adenosine is released from metabolically active cells or generated extracellularly. It is a potent biological mediator modulating the activity of numerous cell types, including neurons, platelets, neutrophils and mast cells, and smooth muscle cells in bronchi and vasculature. Most of these effects contribute to the protection of cells and tissues during stress conditions such as ischaemia. We had previously generated a knockout model for the A_{2a} receptor, which is abundant in basal ganglia, vasculature and platelets, and is considered as a major target for caffeine. In these mice, caffeine was turned into a depressant of exploratory activity. Knockout ani-

mals also scored higher in anxiety tests, were more aggressive, and had increased blood pressure and heart rate. Platelet aggregation was increased (Ledent et al. 1997).

We have pursued the characterization of this model, in collaboration with behavioral pharmacologists of Rouen and other groups. The study investigating the role of the A_{2a} receptor in the anxiogenic effects of caffeine, performed during the preceding year, has been published (El Yacoubi et al. 2000a).

The locomotor stimulatory effects induced by caffeine in rodents have been attributed to antagonism of adenosine A_1 and A_{2A} receptors. Little is known about its locomotor depressant effects seen when acutely administered at high doses. The role of adenosine A_1 and A_{2A} receptors in these activities were investigated in mice. Besides caffeine, the A_{2A} antagonist SCH 58261, the A_1 antagonist DPCPX, the A_1 agonist CPA and A_{2A} receptor knockout mice were used. Caffeine had a biphasic effect on locomotion of wild-type mice not habituated to the open field, stimulating locomotion up to 25 mg/kg doses, while depressing it at 100 mg/kg. In sharp contrast, caffeine dose-dependently decreased locomotion in A_{2A} receptor knockout mice over the whole range of tested doses. The A_1 antagonist DPCPX decreased locomotion of A_{2A} receptor knockouts and CD1 mice. These results suggest that the stimulant effect of low doses of caffeine is mediated by A_{2A} receptor blockade while the depressant effect seen at higher doses under some conditions is explained by A_1 receptor blockade (El Yacoubi et al. 2000b).

The acute motor effects elicited by the A_{2A} receptor antagonists SCH 58261 and ZM 241385 were further investigated in mice. Indeed, an atypical CGS 21680 (A_{2a} agonist) binding site has been described on the basis of the differences of pharmacological activities for the two antagonists. SCH 58261 increased locomotion and rearing with a quick onset, but for a shorter period in mice habituated to the environment than in mice unfamiliar to it. ZM 241385 stimulated horizontal and vertical activities with a slow onset, similarly in naive and in habituated mice. The effects of both antagonists were lost in A_{2a} KO mice, suggesting that 'atypical' CGS 21680 binding sites could be adenosine A_{2A} receptors with a peculiar pharmacological profile (El Yacoubi et al. 2000c).

Knock out mice were also used to examined, in vivo, the influence of the A_{2a} receptor on the state of phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), a potent endogenous inhibitor of protein phosphatase-1, which plays an obligatory role in dopaminergic transmission. The results indicated that, in vivo, the state of phosphorylation of DARPP-32 and, by implication, the activity of protein phosphatase-1 are regulated by tonic activation of D_1, D_2, and A_{2A} receptors, and demonstrate that the adenosine system plays a role in the generation of responses to dopamine D_2 antagonists in vivo (Svenningsson et al. 2000).

Other studies have indicated that deletion of the A_{2a} receptor may lead to region-specific compensatory changes in purine utilisation in brain nuclei associated with autonomic, neuroendocrine and behavioural regulation (Snell et al. 2000a) and suggested that glutamatergic neurotransmission within certain neural pathways involved in autonomic and motor control is altered in the brains of A_{2a}R knockout mice (Snell et al. 2000b).

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

We have continued in collaboration with a number of other groups the phenotypic analysis of our knockout model for the CB_1 receptor, the central receptor for the active compounds of Cannabis and the endogenous ligand anandamide. The role of CB1 in mediating central effects of cannabinoids on body temperature, nociceptive threshold, locomotor activity and blood pressure had been demonstrated previously (Ledent et al. 1999). The finding that CB1-deficient mice have an enhanced capacity to strengthen synaptic connections in the hippocampus, resulting in enhanced memory has now been published (Bohme et al. 2000), as well as the observation that CB1 receptors are essential for adaptive responses produced by chronic morphine but not by chronic cocaine treatment (Martin et al. 2000).

The interactions between the CB_1 cannabinoid receptors and the endogenous opioid system have been further evaluated by assaying a number of well-characterized opioid responses, e.g. antinociception and stress-mediated effects, on mutant mice. The spontaneous responses to various nociceptive stimuli (thermal, mechanical and visceral pain) were not changed in mutant CB_1 mice. Furthermore, the absence of the CB_1 cannabinoid receptor did not modify the antinociceptive effects induced by different opioid agonists: morphine (preferential mu opioid agonist), D-Pen2-D-Pen5-enkephalin (DPDPE) and deltorphin II (selective delta opioid agonists), and U-50,488H (selective kappa opioid agonist) in the hot-plate and tail-immersion tests. In contrast, the stress-induced opioid mediated responses were modified in CB_1 mutants. Indeed, these mutants did not exhibit antinociception following a forced swim in water at 34 degrees C and presented a decrease in the immobility induced by the previous exposure to electric footshock. However, the antinociception induced by a forced swim in water at 10 degrees C was preserved in CB1 mutants. These results indicate that CB1 receptors are not involved in the antinociceptive responses to exogenous opioids, but that a physiological interaction between the opioid and cannabinoid systems is necessary to allow the development of opioid-mediated responses to stress (Valverde et al. 2000).

The knockout mice were used as specificity controls in the characterization of new antibodies directed against the CB_1 receptor, allowing to uncover a more precise distribution of the receptor in the central nervous system. The antibody, developed against the C-terminus of the CB_1 cannabinoid receptor, revealed that, in the hippocampus, a large proportion of boutons in the dendritic layers displaying symmetrical (GABAergic) synapses were also strongly immunoreactive for CB_1 receptors, as were axon terminals of perisomatic inhibitory cells containing cholecystokinin (Hajos et al. 2000).

8. Knock out of orphan receptors.

We have pursued the generation of knock out models for orphan receptors related to neuropeptide receptors. Five genes had been selected on the basis of their distribution in the central nervous system, and of the parallel efforts to characterize their natural agonists. These five genes are JP05/GIR, ACCA, PRPR/GPR10, EST6 and GPR-NGA. The constructs allowing homologous recombination in embryonic stem cells were previously made for ACCA, JP05/GIR and PRPR. The constructs for GPR-NGA and EST6 have been performed this year. Recombined ES cell lines have now been obtained for TBJP05, ACCA, GPR-NGA and the PRP receptor. ES

cell clones have been used in these four cases to generate chimeras. ACCA, GPR-NGA and PRP receptor mutant alleles were transmitted efficiently from some of the chimeras available. These knock-out models, that are apparently healthy, are presently being bred on CD1 and BL6 backgrounds, and the phenotype analysis has been initiated for the ACCA and GPR-NGA models. In a first set of experiments, none of the chimeric mice obtained for the TBJP05 gene transmitted the mutant allele to their progeny. New aggregation experiments have been performed, and a larger number of chimeras were obtained, that are presently being bred. The structure of the mouse JP05/GIR gene, determined in the course of this study, has now been published (De Moerlooze et al. 2000).

9. Molecular mechanisms of development of neuronal connectivity in the cerebral cortex.

The cerebral cortex is one of the most complex and important structures in our brain. In correlation with its elaborate 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 the molecular mechanisms of the generation of specific neuronal connections in the cortex remain largely unknown. We have recently started to characterize the roles of the ephrin/Eph gene family of neuronal signaling molecules (Flanagan and Vanderhaeghen 1998) in the patterning 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 have first shown that one ephrin ligand, ephrin-A5, and several Eph receptors were expressed in the rodent developing thalamus and cortex (Vanderhaeghen et al. 2000). As in lower systems, ligands and receptors displayed complementary gradients of expression, and in vitro assays showed that ephrin-A5 could act as an axon guidance factor for thalamic axons (Vanderhaeghen et al. 2000). Anatomical and functional analysis of ephrin-A5 knock-out mice enabled us to demonstrate a change in shape and dimension of the cortical somatosensory map, resulting in a distortion of body representation in the cortex of the mutant mice (Vanderhaeghen et al. 2000, Prakash et al. 2000). These data constitute one of the first direct evidence for an instructive role of intrinsic genes in the development of a complex neuronal network in the neocortex. Further genetic analysis is required however, because of partial compensation by other as yet unidentified members of the ephrin family , and because of the expression of ephrin-A5 in non-cortical regions of the brain.

In order to test for the effects of a more complete loss of ephrin function specifically in the developing cortex, we have developed a transgenic strategy allowing the overexpression of ephrin antagonists under the control of a cortex-specific promoter. We have focused on two antagonists, a soluble form of ephrin-A5 and a soluble EphA receptor (Flanagan and Vanderhaeghen 1998). These two antagonists were placed under the control of a promoter fragment from the transcription factor Tbr1 (obtained in collaboration with Dr. M. Donoghue from Yale University) that drives specific expression in the developing cortex. These constructs have been used to generate transgenic mice, and the corresponding lines are now being analyzed, that should display a cortex-specific full disruption of ephrin function.

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Report of the Research Group of

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I. Physiology and physiopathology of the basal ganglia system.

Ia. Modulation of ion channels and neuronal excitability by dopamine and adenosine receptors in the striatum (patch clamp ands extracellular recording studies)

We previously shown that in striatal neurons, dopamine acting at the D1 receptor reduces the amplitude of the voltage-dependent sodium current. This effect involves activation of the cAMP-dependent protein kinase (PKA) and at least partially through the phosphorylation of DARPP-32 the inhibition of phosphatase. This led to a depression in striatal neuronal excitability by increasing the threshold for generation of action potentials. We also showed using a molecular model of the channel gating mechanism that this results from an acceleration of the inactivation from the open state.

It has been clearly demonstrated in other cellular models, that besides the cAMP-PKA pathway, other intracellular signalling cascades such as the one initiated by phospholipase C (PLC) are also able to modulate the voltage-dependent sodium current. This pathway give rise to the production of two intracellular messengers: diacylglycerol (DAG) an activator of protein kinase C (PKC) and inositol trisphosphate (IP3). Since, both dopamine and adenosine acting at specific receptor subtypes may stimulate this pathway, we evaluated the involvement of these two second messengers in the regulation of sodium current in striatal neurons in culture. We have first tested the effect of adenophostine A, an activator of IP3 receptor and observed that this resulted in a 15% increase in sodium current amplitude. This effect was totally abolished when cells were loaded with a solution containing a Ca^{++} buffer such as EGTA or BAPTA. We hypothesised that the IP3-mediated intracellular Ca^{++} release would result in the activation of calcineurin (protein phosphatase 2B) and therefore in the dephosphorylation of sodium channels. Conversely, the activation of PKC by phorbol esters such as TPA led to a 30 % decrease in sodium current amplitude in same neurons. Altogether, this suggests that the activation of the PLC pathway may result in differential modulations of sodium current. Sets of experiments are now in progress to evaluate the physiological relevance of each part of this PLC pathway following stimulation of specific adenosine and dopamine receptors.

We have also pursued the study of the modulation of the corticostriatal transmission by dopamine and adenosine in the accumbens nucleus using patch clamp and extracellular recording techniques on brain slices. We have first focused ourselves on the possible modulation of synaptic transmission at this glutamatergic synapse by adenosine receptors using the standard field potential recording technique on brain slices from wild-type and A_{2A} receptor-deficient mice ($A_{2A}r^{-/-}$) [9]. Neither the stimulus-response relationship nor paired-pulse facilitation were altered in the mutant mice. In both genotypes, the activation of A_1 receptors reduced the field excitatory postsynaptic potential (fEPSP) slope to a similar extent. In wild-type slices, activation or blockade of A_{2A} receptors did not modify the synaptic transmission. Moreover, a long lasting pre-activation of these A_{2A} receptors did not influence the A_1 receptor-mediated reduction in fEPSP slope. Altogether, these data provide the evidence that the A_{2A} receptor-mediated pathway is not involved in the regulation of the basal or the A_1-modulated AMPA receptor-mediated excitatory synaptic transmission in the accumbens. Long term potentiation (LTP) of the AMPA receptor-mediated synaptic transmission could be elicited in both wild-type and A_{2A} receptor-

deficient mice. However, in the mutant mice, this LTP developed differently with a significant lower potentiation level during the first 20 minutes and a final potentiation value lower but not significantly different from that obtained in wild-type. This demonstrated that A_{2A} receptor is implicated in events directly or indirectly related to the early phase of LTP. Experiments were now carried out in order to elucidate the mechanism(s) leading to this alteration. Preliminary results pointed to the involvement of the cAMP-PKA cascade.

Ib. Modulation of gene expression in the basal ganglia

We previously reported that the induction in striatal immediate early genes (IEG) expression following the injection of a single dose of caffeine or the A_1 antagonist, DPCPX involved different mechanisms in striatonigral and striatopallidal neurons through blockade of A_1 receptors: a stimulation of dopamine release for the former and activation of acetylcholine and/or glutamate release in the latter [Dassesse et al., 1999 and 17].

We have now analyzed the role of the A_{2A} receptor in the control of both basal and caffeine-induced IEG striatal expression. The basal IEG levels and the acute effects of caffeine and DPCPX on IEG expression were evaluated on wild-type and A_{2A}-receptor deficient mice [10]. In situ hybridization and immunocytochemical experiments showed that the basal expression of IEGs was significantly decreased in striatum, cerebral cortex and hippocampus of mutant mice and that the dose-response effect of caffeine on IEGs expression was biphasic in the wild-type and monophasic in the mutant mice. To understand the mechanisms leading to these changes in basal striatal gene expression, we undergo the analysis of basal neurotransmitter release in the striatum of wild-type and A_{2A}-receptor deficient mice by using microdialysis and HPLC determination of neurotransmitter concentration in collaboration with the laboratory of Neuroendocrinology and Immunological Biotechnology, KUL (Prof. F. Vandesande). The results unexpectedly showed a significant 3-fold increase in extracellular glutamate concentration in the striatum of A_{2A}-receptor deficient mice. In contrast, the level of N-methyl-D-aspartate receptors and glial glutamate transporters GLT-1 and GLAST were indistinguishable between A_{2A} knockout and wild-type mice. Since several arguments suggest a dysfunction of the dopaminergic system, determinations of the extracellular striatal dopamine concentrations in both genotypes are currently performed.

To further characterize the adaptive changes in striatal gene expression suspected in $A_{2A}r^{-/-}$ mice, the expression of substance P, enkephalin, GAD65, GAD67, dopamine D_2, D_1 receptors and adenosine A_1 receptors was analyzed. The expression of A_1 receptors both at the mRNA (in situ hybridization) and the protein (binding) levels was unaltered. Conversely, the basal expression of substance P, enkephalin, GAD65, GAD67, dopamine D_2 and D_1 receptors were dramatically modified. Evaluation of the effects of chronic treatments with different doses of caffeine or DPCPX on striatal also demonstrated marked differences between wild-type and mutants mice. Altogether, our results would suggest the existence of important changes in the basal ganglia physiology in A_{2A}-receptor deficient mice and are highly suggestive of an impairment in dopaminergic transmission.

We have also analyzed the effect of manipulations in the adenosinergic or dopaminergic transmission on the nociceptin expression in the septum [2]. Indeed, most effects of nociceptin are related to blockade of stress and anxiolytic-like effects and it is highly expressed in septal

nuclei, which are involved in response to stressful situations. On the other hand, dopamine and adenosine may have modulatory effects on stress behaviour by acting on septal neurons and this could be related to a modulation in nociceptin gene expression. No difference in nociceptin expression was observed between wild type and A_{2A} receptor-deficient mice. In both genotypes, chronic caffeine treatments at different doses did not significantly modify nociceptin expression. A 6-hydroxydopamine-induced dopamine depletion was also without any effect. These results demonstrated that dopamine and adenosine are not involved in the regulation of septal nociceptin expression in spite of the involvement of these three neurotransmitters in stress and anxiety behaviours.

Ic. Striatal subpopulations specific gene inactivation in conditional transgenic mice.

As pointed out in the intermediary report of 1999, we initiated in mid 1998, the development of a molecular biology subunit in the lab. In the basal ganglia field, the construct of a *floxed* (for flanked by loxP) GAD67 mice and mice expressing the CRE recombinase on the control of gene(s) promoter specifically expressed by either striatopallidal or striatonigral neurons have been initiated. These two subpopulations and the different loops taking rise from them are well recognized but their differential roles are poorly understood. Crossing of *floxed* GAD67 and CRE mice will result in cell-specific inactivation of this enzyme producing the main neurotransmitter (GABA) of these neurons and this will allow to shed more light on their differential role(s) in the basal ganglia physiology. This would not be possible using a classical KO strategy since GAD67$^{-/-}$ KO mice died at birth and the inactivation of GAD67 is required since the inactivation of GAD65 did not change the brain GABA content. The resulting mice will be analyzed using the techniques available in the lab. and in collaboration for behavioural analysis and *in vivo* electrophysiology.

The first construct designed to allow the specific expression of the CRE recombinase specifically in striatopallidal neurons has been achieved last year and was injected in the first semester of 2000 in mouse oocytes to generate transgenic mice. The generated founders are currently expensed and for some of them the analysis has been started by using anti-CRE antiserum or by crossing them with mice expressing LacZ gene following by a floxed *stop* codon, in order to select the one(s) exhibiting the selective expression of CRE. These steps are currently in progress.

As stated last year, our CRE mice would also be used in other collaborative studies by crossing them with *floxed* genes expressing mice to inactivate these genes selectively in striatopallidal or striatonigral neurons. We have already established a collaboration with a German group who produced both a mouse line expressing CRE in another specific striatal cell population and mice expressing floxed genes.

Id. Selective striatal subpopulations neuronal death in Huntington's disease

Striatopallidal and striatonigral neurons are also differentially affected in pathologies of the basal ganglia system through mechanisms remaining unknown. Based on our knowledge of the neurochemistry and physiology of these neurons; we have started to address this question. We have developed in the lab. an experimental model of Huntington's disease in rat or mouse by using subchronic injection of 3-nitropropionic acid. Our firs aim was to prove the high repro-

ducibility of this model in terms of lesion. We demonstrated indeed that the infusion of this toxin using osmotic minipump results in specific striatal lesion which exhibit highly reproducible parameters regarding its size and its topography as well as regarding its behavioural consequences [13]. This model is therefore now perfectly suitable to study the precise time-course of neurochemical alterations in each neuronal subpopulations.

II. Functional and morphological characterization of different proteins involved in the intracellular calcium homeostasis.

IIa. Cerebellar physiology in transgenic mice lacking calretinin.

In the cerebellum, the parallel fiber-Purkinje cell synapse can undergo long-term synaptic plasticity implicated in motor learning and resulting from variations in intracellular calcium concentration. This parallel fiber pathway contained high level of calretinin. We previously showed that $Cr^{-/-}$ are impaired in tests of motor coordination suggesting functional deficits in cerebellar pathways. Calbindin-D28K is highly saturated in calcium in $Cr^{-/-}$ Purkinje cells as compared to $Cr^{+/+}$ mice, supporting an impairment in intracellular calcium homeostasis. The firing behaviour of $Cr^{-/-}$ Purkinje cells analyzed in alert mice was severely affected whereas, in cerebellar slices, excitatory synaptic transmission from parallel fibers or climbing fibers to Purkinje cells was unaltered despite the absence of calretinin in granule cells [Schiffmann et al. *Proc. Natl. Acad. Sci.* USA, 96: 5257-5262, 1999 and 3]. These results pointed out that somehow, calretinin plays a major role at the network level in cerebellar physiology.

Several lines of research have been developed from mid 1999 to understand the mechanisms(s) leading to the dysfunction of the cerebellum of these mice. Not only we hope to identify it(them) but we also believe that this identification will unmask unknown aspects of the cerebellar circuit models.

Since the granule cells are the major calretinin-positive cell in the wild-type animals, their electrophysiological properties including the $[Ca^{2+}]_i$ homeostasis are analyzed *in vitro* on brain slices using the patch clamp technique. Although technically difficult on granule cells, the « perforated patch » configuration of the patch clamp technic was recognized as the most useful to answer our questions in order to limit the perturbation of the granule cell physiology. This allowed to reveal alterations in intrinsic electrophysiological properties of granule cells in $Cr^{-/-}$ mice as compared to wild-type. A significant increase in granule cell excitability expressed as an increase in action potential frequency per unit of injected current as well as a significant reduction in the action potential duration and an increase in the speed of repolarisation phase of the action potential were observed in $Cr^{-/-}$ mice. A mathematical model of the electrical activity of granule cell is currently developed in order to firmly establish the link between the experimental observations and the absence of cytosolic calretinin. To address more specifically the putative alterations in granule cell physiology, their responses to the stimulation of their afferences, the mossy fibers, are analyzed in brain slices of $Cr^{-/-}$ and wild-type mice. The involvement of Ca^{++} homeostasis is central in long term modifications of synaptic efficacy at many synapses. Long term potentiation at the mossy fiber to granule cell synapse will therefore be compared in $Cr^{-/-}$ and wild-type mice.

To demonstrate whether or not the observed cerebellar dysfunction in calretinin-deficient mice actually results partially or completely from its absence specifically in granule cells, the following mice are in construction and will be analyzed such as previously published [Schiffmann et al. *Proc.*

Natl. Acad. Sci. USA, 96: 5257-5262, 1999 and 3 and see above]. The calretinin expression will be selectively rescued in the granule cells by directing gene expression to cerebellar granule cells using a well characterized $GABA_A$ receptor $\alpha 6$ subunit construct and generation of transgenic mice. The same construct is used to express an antisens calretinin gene resulting therefore in the cell-targeted inhibition of calretinin expression in granule cells of wild-type mice. This will allow to see whether or not this decreased expression somehow mimic the phenotype observed in $Cr^{-/-}$ mice. We have kindly obtained this $GABA_A$ receptor $\alpha 6$ subunit construct from Prof. Wisden. Constructions have been realized in the lab. in which this promoter is followed by the mouse calretinin gene in sens or antisens direction. These constructions have been injected in the first semester of 2000 in mouse oocytes to generate transgenic mice. The generated founders are currently expensed and for some of them the analysis has been started using anti-calretinin immunohistochemistry, in situ hybridization and RT-PCR in order to select the one(s) exhibiting the selective expression of calretinin or calretinin antisens mRNA. These steps are currently in progress.

III. Physiopathology of enteric nervous system and interstitial cells of cajal iin gastrointestinal motility in human and mouse. Morphological and functional approaches.

1. Structural characterisation of interstitial cells of Cajal (ICC) and their relationship with nerve fibres and other cell types in the human and murine GI tract, by means of immunohistochemistry, confocal microscopy and electron microscopy [6, 7, 11, 15]; in collaboration with Dr Juri J Rumessen, Copenhaghen (DK) , Professor Jean-Jacques Panthier, Maisons-Alfort (F) , Professor Guy E. Boeckxstaens, Amsterdam (NL) and Dr Luc Andries, Beerse (B). Furthermore, a novel collaboration with Prof. Panthier has been initiated on an original mutant mouse strain (Pm), featuring a marked organomegaly of the GI tract. Our recording in vitro suggests a significant increase in the intestinal slow wave (pacemaker) frequency. The morphometrical analysis of the intestinal has been initiated. The mode of inheritance of the trait and the identification of the genetic defect involved are under progress.
2. Morphological studies of the neuromuscular system in the human GI tract in health and disease by means of pathological staining, immunohistochemistry and retrograde labelling in vitro [4, 14, 16].
3. Identification of a novel oncogenic mutant of the proto-oncogene KIT in a family with multiple gastrointestinal stromal tumors [5]. Molecular and cellular analysis of several oncogenic mutants in murine Ba/F3 cell lines, by means of gene sequencing, single strand conformation polymorphism analysis, site-directed mutagenesis, stable expression in mammalian cell lines, phosphorylation studies, immunoprecipitation, western-blotting, proliferation assay, chemotactic assay, calcium influx measurement.
4. Molecular approach to identify genes involved in the mechanism of the intestinal pace-maker by means of a "PCR-select™ "substractive strategy (including PCR-based techniques, subcloning and sequencing of PCR products, northern blotting) comparing cDNA pools obtained from the intestinal muscle coats of normal mice and from their ICC-deficient littermates, respectively. A collaborative research program with Janssens Research Foundation , Beerse, (B) has been concluded on molecular aspects of the intestinal pacemaker, providing support for the microarray technology and for the production of transgenic mice expressing the reporter

gene EGFP under control of the c-kit promoter, for the direct identification of living ICC in situ and in culture. Creation of a core facility for laser microdissection and laser pressure catapulting ™ (PALM system). Evaluation of the system and preliminary experiments have been performed in other Universities. The system will be used in our research program to validate candidates identified by the substractive technique described above.

IV Involvement of the phosphoinositides cascade in the neural stem cells proliferation and differentiation

The germinative ventricular zone (VZ) of embryonic brain contains neural lineage progenitor cells that give rise to neurons, astrocytes and oligodendrocytes. The ability to generate neurons persists at adulthood in restricted brain areas. During development, many growth factors exert their effects by interacting with tyrosine kinase receptors and activate the phosphatidylinositol-3 kinase and the Ras/MAP kinase pathways. By its ability to modulate these pathways, the recently identified inositol polyphosphate 5-phosphatase 2 (SHIP2) has the potential to regulate neuronal development. Using *in situ* hybridization, we showed that SHIP2 mRNA was highly expressed in the VZ of brain and spinal cord and whereas no significant expression was seen differentiated fields [8]. This restricted expression was maintained from E12 to birth. In the adult brain, SHIP2 was mainly restricted in structures containing neural stem cells such as the anterior SVZ, the rostral migratory stream and the olfactory tubercle. The specificity of SHIP2 expression in neural stem cells was further demonstrated in the adult by the dramatic increase in SHIP2 signal in N-CAM-deficient mice, which present an accumulation of progenitor cells in the anterior SVZ and the rostral migratory stream. In collaboration with Dr. B. Rogister from the Center for Cellular and Molecular Neurobiology, University of Liege, the expression of the SHIP2 protein was assessed using Western blot analysis on neurosphere cultures either in proliferation or differentiation conditions. It was abundantly expressed in proliferating neurospheres whereas it was barely detectable in differentiated neurospheres, confirming therefore the specific expression in undifferentiated neural stem cells. The correlation between the pattern of SHIP2 expression in the brain and the proliferative and early differentiative events suggests that the phosphatase SHIP2 may have important roles in neural development. Experiments are now started in order to address the physiological relevance of this specific expression in the neural cell proliferation and/or differentiation process.

V. 5-HT1A receptors in the KA model of limbic epilepsy in the rat

In collaboration with the PET/Biomedical Cyclotron Unit (ULB), the correlation between hippocampal neuronal loss and *in vivo* 5-HT1A receptors detection as been assessed in the kainic acid (KA) model of limbic epilepsy in the rat [12]. A high density of 5-HT1A receptors is present in pyramidal hippocampal cells and they can be mapped *in vivo* using ^{18}F-MPPF. We tested the hypothesis of a correlation between MPPF binding and post-epileptic neuronal loss in the hippocampus. Determination of MPPF binding in the brain was combined with a quantification of neuronal loss using DNA labeling with propidium iodide and confocal microscopy in brains of rats sacrificed 1 hour to 240 days after KA injection. An initial relative decrease in hippocampal MPPF binding from the 1st to 6th days was followed by an increase between the 6th and 30th days. This effect was observed in rats who showed hippocampal neuronal loss as well as

in those who did not. In rats sacrificed outside this initial period of time, correlation between hippocampal MPPF binding and neuronal loss was statistically significant. The increase of MPPF binding observed from the second week after KA injection suggests an unexpected epilepsy-induced increase of 5-HT1A receptors in the hippocampus.

VI. Axonal transport of potassium channels

The recent cloning, functional expression and brain localisation of two new potassium channels TREK and TRAAK led us to examine whether both channels are present in peripheral nerves and can move along axons by means of axonal transport mechanisms. Using specific antibodies directed against TREK and TRAAK peptides, we found that immunoreactivity for both potassium channels accumulates above and below a ligature in rat sciatic nerves [1]. The process was rapid and bi-directional indicating that the channels are associated with vesicles.

VII. CCR5 chemokine receptor trafficking

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

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Book Chapters

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Positional cloning of the neuronal and spinal form of Charcot-Marie-Tooth disease

Inherited peripheral neuropathies have a prevalence of 1/2500 and are grouped into the hereditary motor and sensory (HMSN), motor (HMN) or sensory (HSN) neuropathies. Charcot-Marie-Tooth (CMT) disease is the most common form of inherited peripheral neuropathies. Clinical, neurophysiological and histopathological criteria are used to differentiate CMT into demyelinating CMT or CMT1, neuronal CMT or CMT2, and spinal CMT. CMT1 and CMT2 belong to the HMSN, while spinal CMT belongs to the distal HMN. Up to now at least 26 loci and 9 genes for different inherited neuropathies have been assigned and identified in the human genome (De Jonghe et al., 2000b).

Genetic linkage studies mapped four CMT2 loci i.e. CMT2A, CMT2B, CMT2D and CMT2E at chromosome 1p35-p36, 3q13-q22, 7p14 and 8p21 respectively. CMT2C is an as yet unmapped locus (De Jonghe et al., 2000b). We reported a large Italian CMT2 family linked to the CMT2A locus on chromosome 1p35-p36. Based on previously reported genetic data and the recombination events in our Italian family we could refine the CMT2A region from 16 to 10 cM. The clinical phenotype is uniform and similar to that reported in other CMT2A families, confirming intra- and inter-familial uniformity of the CMT2A phenotype. This CMT2A phenotype is distinct from that observed in CMT2 families associated with mutations in *peripheral myelin protein zero (MPZ) or connexin 32 (Cx32/GJB1).* In these latter cases, nerve conduction velocities (NCVs) can initially be slightly reduced or nearly normal but in elderly patients NCVs may drop to 25 m/s. The NCV range is much broader than in the Italian CMT2A family, and the lower limit is much lower. A sural nerve biopsy indicated a process of de- and remyelination besides axonal involvement. Many genes are known to be located in the telomeric region of chromosome 1p. So far no obvious candidate genes have been identified. The study of this CMT2A family will contribute to the identification of the CMT2A gene (Muglia et al., 2000).

Recently a fourth CMT2 locus (CMT2E) was mapped to chromosome 8p21 in a single CMT2 pedigree from Mordovia, Russia. Subsequently a c.998A>C transversion mutation resulting in a Gln333Pro in the first exon of the *neurofilament light (NEFL)* gene was found to show complete co-segregation with the disease phenotype. We screened 40 unrelated patients diagnosed as CMT2 or intermediate CMT for the presence of mutations in the *NEFL* gene. Via DHPLC (denaturing high performance liquid chromatography) we detected an heteroduplex pattern in one of the NEFL-PCR fragments in the proband of a Belgian CMT family. DHPLC analysis demonstrated the same heteroduplex pattern in all patients from the same family, but not in their unaffected relatives. We subsequently sequenced DNA samples of three patients and found a double missense mutation at positions 22 and 23 from CC to AG in the first exon of NEFL (c.22C>A+23C>G). This mutation creates an amino-acid change from proline to arginine at codon 8 (P8R). This mutation was absent in 80 normal controls suggesting that this sequence variation is not a rare polymorphism. Linkage analysis with the Pro8Arg mutation in family CMT-56 resulted in a two-point LOD score of 3.61 in the absence of recombinants. The NEFL Pro8Arg missense mutation most likely destabilizes the head domain of the NEFL protein. The patients in the Belgian family present with a classical, although rather severe CMT phenotype

with a disease onset in the second decade of life. The NVCs are sometimes severely slowed and patients could be classified as CMT1 based on NCVs alone. It is important to note that amplitudes of the CMAP are always severely reduced suggesting that the slowing is, at least partially, due to loss of fast conducting axons. Our data suggest that also patients and families that are diagnosed as CMT1 should be screened for mutations in the *NEFL* gene once mutations in the CMT1 genes have been excluded (De Jonghe et al., 2000a).

CMT2B is a rare disorder belonging to the axonal CMT syndromes which is clinically characterized by marked distal muscle weakness and wasting as well as a high frequency of foot ulcers, infections and amputations. So far only two families with this disorder have been described in which molecular genetic studies have shown evidence of autosomal dominant inheritance with linkage to chromosome 3q13-q22. We reported a large Austrian family presenting with the typical clinical features of CMT2B. Eight family members were definitely affected upon clinical and electrophysiological examination and the majority revealed pronounced distal muscle wasting and weakness as well as prominent sensory abnormalities, which were frequently complicated by infections and amputations. The patients have normal or slightly to moderately slowed motor NCV, markedly reduced CMAP with chronodispersion, and absent or reduced amplitudes of SNAP. The molecular genetic study demonstrates linkage to chromosome 3q13-q22. Haplotype analysis in affected individuals indicates that the CMT2B locus is located between the flanking markers D3S1589 and D3S1549 representing a region of 10 cM. This family is the third CMT2B family reported so far and confirms the existence of the CMT2B locus on chromosome 3q13-q22, which is responsible for a clinically and electrophysiologically homogeneous disorder with prominent distal muscle weakness and wasting, and ulcero-mutilating features. Marked sensory disturbances and the high frequency of foot ulcers, infections, and amputations in our patients seem to be typical for CMT2B (Auer-Grumbach et al., 2000a). Direct amplification and sequencing of genomic DNA from affected individuals was used to assess *SOX14* as a candidate gene. No changes of the SOX14 sequence between patients and normal controls were found, excluding mutation in the *SOX14* coding region as the genetic cause of CMT2B (Hargrave et al., 2000).

We also reported detailed clinical, electrophysiological and genetic data of an other large Austrian family with ulcero-mutilating neuropathy, sensory loss and amputations. Linkage analysis with chromosomal markers representing the HSN I and HMSN IIB loci excluded these gene loci in our family. These findings therefore indicate the existence of a third gene locus in autosomal dominant inherited ulcero-mutilating neuropathies, showing that these neuropathies are genetically heterogeneous (Auer-Grumbach et al., 2000b).

In 1996 we assigned the disease locus for autosomal dominant hereditary motor neuropathy type II (distal HMN II) within a 13 cM interval at 12q24.3. We constructed a physical map of the distal HMN II region based on yeast artificial chromosomes (YACs), P1-artificial chromosomes (PACs) and bacterial artificial chromosomes (BACs) using a sequence-tagged-site (STS) content mapping approach. The clone conting covers 5 Mb and 81 STS markers including 25 known STSs and short-tandem-repeats (STRs), 33 new STSs generated from clone end-

fragments, 18 expressed-sequence-tags (ESTs) and 5 known genes, were located on the contig. The construction of an integrated clone contig of 12q24.3 allowed fine mapping of several genes and ESTs of which exact localisation within the distal HMN II region was unknown. An advanced BLAST search on all markers used in the contig mapping approach, revealed that some ESTs showed high homology to known human genes. This enabled us to locate a few positional candidate genes for distal HMN II which are currently screened for pathogenic mutations (Irobi et al., 2000).

Gigaxonin is a novel cytoskeleton protein and member of the kelch repeat superfamily. Mutations in gigaxonin lead to Giant Axonal Neuropathy (GAN), a recessive motor and sensory neuropathy of the central and peripheral nervous system, mapping to chromosome 16q24.1. GAN patients show a distortion of nerve fibers due to axonal swellings by the accumulation of neurofilaments (Timmerman et al., 2000).

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I. FMRI in Awake Behaving Macaques Reveals Mechanisms of Visual Motion Processing

Introduction

To construct generalized models of 'primate' visual cortical processing, it has been necessary to draw arbitrarily from either human or macaque reports, according to what data were available on a given topic. However, apparent differences between human and macaque visual cortex have been reported with increasing frequency. To the extent that such species differences are confirmed, these 'general' models of primate visual cortical function will need to be modified accordingly. The problem is that human research and macaque research have been based on largely different techniques. Thus any apparent differences between macaque and human visual cortex could actually be due to either 1) genuine species differences, or instead to 2) artifactual differences arising from the different techniques used.

One technique which is increasingly used to reveal human visual cortical function is fMRI (functional magnetic resonance imaging). We used fMRI to map activity in visual cortex of the macaque. To make our experimental situation as similar as possible to the human fMRI studies, we trained the monkeys to fixate a visual stimulus throughout the period of fMRI scan acquisition. Such fMRI studies, from monkeys which were awake *and fixating*, have not been reported in detail previously– although several reports have demonstrated that fMRI is feasible in monkeys (Logothetis et al., Stefanacci et al., 1998; Vanduffel et al., 1998). Such 'linking' experiments will help bridge the information gap between all the different kinds of experiments on primate visual cortex (e.g. macaque single units, human fMRI, human psychophysics, monkey connectional data, etc.).

We used fMRI to study visual motion processing, using stimuli which were either static, flickering, or coherently moving. This furnished several complementary measures of motion sensitivity, which could help distinguish the cortical visual areas which are functionally homologous in macaques and humans. Another question dealt with the progressive processing of motion at different levels in the cortical hierarchy.

Results

Two macaque monkeys were implanted with a near-conventional headset, using MR-compatible materials. The monkeys were then adapted to restraint in a natural 'sphinx' position, facing parallel with the bore of a conventional MR scanner (Siemens 1.5 T, horizontal bore), equipped with echo-planar imaging. For a juice reward, monkeys continuously performed an orientation discrimination experiment ('fixation task') while in the scanner, based on a very small (5 x 18 min arc) single bar positioned at the center of the visual display screen. The small size of this orientation target ensured that this task could only be done using central fixation (see below). Different visual stimuli, (irrelevant to the ongoing fixation task) were presented in the remaining portion of the display screen (14 degrees in diameter). These stimuli included random dots in three motion-related conditions (planar motion, stationary flickering, and stationary). Each random dot condition was presented in 24 second blocks (epochs), in alternation with 24-second blocks of a uniform grey screen (to track baseline MR levels). The monkeys made task responses by tripping a light-activated switch. Analysis of the fMRI data was performed using SPM99 and FreeSurfer.

Validation tests, V5/MT+ and V1

As one might expect, the three motion-related conditions produced different fMRI effects in correspondingly different parts of the brain. First we needed to confirm that fMRI could localize macaque V5/MT+. Such localization information is shown in Figure 1, for area V5/MT+, from both monkeys tested (M1 and M2). The statistical maps represent the significance of the regional differences between the fMRI responses to the moving stimuli and those to stationary stimuli. This moving-stationary stimulus comparison is the standard for functional localization of hV5/MT+ in human visual cortex. In both monkeys, there was significant motion-sensitive activity in the fundus and posterior bank of the dorsal superior temporal sulcus, bilaterally. Although there were small variations in position with replications and between hemispheres, the fMRI-defined 'V5/MT+' occupies rather precisely the location of macaque V5/MT (perhaps including the small adjacent area, MST), as based on previous techniques. The fact that bilateral activation foci were seen in the same cortical region, in both monkeys, suggests that our fMRI approach offers good inter-subject reliability.

These statistical maps were clarified by examining the averaged fMRI time courses from 5 scans produced by the moving, the flickering, and the stationary stimuli. As expected, area V5/MT+ showed robust fMRI responses to moving stimuli, but very little response to the stationary stimuli (see Figure 2A). In contrast, primary visual cortex (V1) showed a more general response, responding fairly well to all three of our stimuli (see Figure 2B).

Range of motion-sensitive areas

The finding of predictable fMRI responses in these well-studied areas encouraged us to test functional activity in additional areas of macaque visual cortex, which received less study previously. Initially we restricted our sampling to areas which showed significant motion-sensitive activity (moving-minus-stationary). These areas were: V1, V2, V5/MT+, mSTS and IP. Though a moderate percentage of direction-selective neurons have been reported in area V3, our fMRI did not reveal motion-sensitive activity in V3. The lack of MR signal in V3 may be due to partial-volume MR sampling, relative to this very thin cortical area (< 3 mm width).

Ventral to V5/MT+, but still within the superior temporal sulcus, we found an additional focus of motion-sensitive activity, which we have given the provisional name 'mSTS' (middle STS). This activity was bilateral, and well-separated from that in V5/MT+. Averaged over the 4 hemispheres, the mean distance between the local maxima in MT/V5+ and in mSTS equalled 7.5 mm.

Flicker Reduction

One of our hypothesis was that fMRI responses to purely temporal probe stimuli (i.e. the flickering stimulus) would be systematically reduced at correspondingly higher-tier levels of the visual hierarchy–relative to the response to spatio-temporal probe stimuli (i.e. the moving stimulus). These results have been presented for the two monkeys in Figure 3A. All the motion-sensitive areas are arranged on the X-axis according to their level in the visual cortical hierarchy. The amplitudes shown represent the level of flicker reduction, relative to the response to planar motion stimuli in that same area.

In V1 (hierarchical level #1), the response to purely temporal stimuli is almost as high as that to spatio-temporal stimuli in V1 (14.5% flicker reduction, see Figure 3A). However at pro-

figures 1, 2A, 2B & 3A, see pages 195-196

gressively higher levels of the cortical hierarchy, the levels of responses to these two stimuli diverge accordingly. At the highest levels tested (#7 / #8) there is very little response to the flickering stimuli compared to stationary stimuli (59.1 and 99.8% flicker reduction in mSTS and IP, respectively), although responses to the planar motion, again compared to stationary stimuli, remain quite robust. Thus the 'flicker reduction' predicted by models of visual motion processing shows up quite clearly in these data

Discussion

Macaques and humans:

One of our goals was to compare brain activation in macaques and humans, using a common measurement technique. We chose fMRI as the common technique for multiple reasons. fMRI is non-invasive, and it yields maps of brain activity throughout the brain, with unlimited potential for repeated mapping in the same subject(s), using new and different stimuli as experimental questions evolve. Using this common mapping technique, we found that many cortical areas (e.g. V1, V5/MT+) appeared functionally equivalent in humans and macaques, at least in the tests we performed here.

Our results also raised related questions. For instance, we found distinct motion-specific foci ('mSTS') located bilaterally in the superior temporal sulcus, 7.5 mm antero-ventral to V5/MT+. Motion-sensitive activity has also been seen in this region of the STS, in deoxyglucose-labeling experiments in awake behaving macaques (Orban et al., 1997). An area ('FST') has been previously defined in that general vicinity of macaque cortex based on anatomical connections from V5/MT+, so it is possible that our 'mSTS' (as defined functionally) is actually part of FST (based on anatomical connections). Alternatively, 'mSTS' may represent the posterior portion of the superior temporal polysensory area (STP), in which complex motion responses have been reported (Oram et al., 1993).

Flicker reduction:

In monkeys, the response to flickering stimuli gradually decreased as we sampled from hierarchically higher areas, whereas a response to planar motion remained robust throughout the same areas (see Figure 3A). This monkey-based effect appears similar to previous fMRI reports in humans (Sunaert et al., 1999). However in humans, the cortical connections and resultant hierarchy remain unknown.

By instead doing this experiment in macaques, we were able to demonstrate a systematic and progressive change in the flicker response with respect to the cortical hierarchy. The single cell study of Qian et al. (1994) showed flicker reduction in area V5/MT neurons, which was similar to what we observed here. That study (Qian et al., 1994) provided an important clue to the implementation of the flicker reduction: it might arise from strong suppression by motion in the non-preferred direction. Hence, the flicker reduction is in excellent agreement with the motion modeling studies which imply an opponent stage (e.g. van Santen and Sperling, 1985), and with psychophysical studies reporting interactions between directional mechanisms (e.g. van Santen and Sperling, 1985).

An alternative explanation for the gradual increase in flicker reduction might be an increase in receptive field size at progressively higher levels of the cortical hierarchy. At higher

figure 3A, see page 196

levels of the visual processing hierarchy, receptive fields become progressively larger, so that calculations of spatio-temporal variations across them become increasingly coherent.

In our data, the reduction increases progressively along the visual pathway. This agrees with reports that detection of moving patterns (which likely depend on early visual stages) reveals some independence between opposing direction mechanisms. This also might allow transparency to occur at middle stages such as MT/V5 (Qian et al., 1994a).

II. Contrast agent enhanced FMRI in Awake Behaving Macaques

A monocrystalline iron oxide nanoparticle (MION) was employed as an MRI contrast agent in awake, behaving macaques in order to improve the sensitivity for functional mapping of the visual cortex. No adverse behavioral effects were observed following injection of 8-12 mg iron per kg body weight, even after repeated injections on successive days in the same animal. The use of contrast agent significantly increased the quality of functional brain maps: the contrast to noise ratio for signal changes in primary visual cortex was increased by a factor of ~ 4 relative to the common blood oxygen level dependent (BOLD) technique. The temporal response of signal weighted by cerebral blood volume after injection of agent differed from that of BOLD signal, whereas the spatial localization of statistical foci derived from the two methods was similar at the 3 mm resolution used in this study.

Introduction

Functional MRI based upon blood oxygen level depend (BOLD) signal has become a routine tool of human neuroscience, and several groups have previously reported fMRI results in non-human primates using BOLD contrast (Stefanacci et al., 1998; Logothetis et al., 1999; Vanduffel et al., 1998). While BOLD signal has proven to be a powerful technique in human and animal studies, small signal changes at low magnetic fields are a limiting factor for detecting subtle changes in local perfusion or for obtaining high spatial resolution. MRI contrast agents with long blood half lives provide an alternative contrast mechanism in animal models. In particular, dextran-coated iron oxide agents (Weissleder et al., 1990) have found utility in a wide variety of animal research and now are being tested in human clinical trials.

For functional brain mapping, the potential of exogenous contrast agent is three fold. First and foremost, this method has been shown to provide a large increase in the sensitivity for detection of brain activation relative to BOLD signal in anesthetized rodents (Mandeville et al., 1998). Secondly, functional signal changes are larger in brain parenchyma than in large vessels, as defined by the blood volume fraction in each voxel, after injection of an appropriate amount of agent. This reverses the order for BOLD signal, which exhibits the largest signal changes at high venous blood volume fractions. Finally, large doses of contrast agent overwhelm effects from deoxyhemoglobin, so T2 relaxation predominantly reflects changes in cerebral blood volume (CBV). fMRI studies are thus uniquely suited for spatial-temporal comparisons of the physiology of CBV in relation to BOLD signal, which contains a significance dependence on blood volume.

The agent used in this study was a monocrystalline iron oxide nanoparticle (MION) that was manufactured at the Massachusetts General Hospital (Charlestown, MA, USA).

MION was injected into the femoral vein prior to placing the macaque inside the magnet. The injected dose, 4-12 mg-Fe/kg, was based upon previous results in rats, as well as estimates of the signal drop in monkeys obtained by comparing absolute signal values across imaging sessions.

Analysis of functional sensitivity

The percentage MR signal change is compared before and after the injection of MION using SPM99. In each case, the block stimulus paradigm was convolved with a hemodynamic response function (HRF) to generate a maximum likelihood estimation of the vascular response to serve as a reference function for determination of the Z statistic. Since the temporal shapes of signal evolution were qualitatively different before and after injection of MION, different hemodynamic response functions (HRF) were used for analysis of BOLD and CBV-weighted signal. When no contrast agent was used, the analysis employed a standard BOLD HRF described by a gamma variate function.

Results

Behavioral Response to MION

MION contrast agent was injected into three animals at doses of 8-12 mg-Fe/kg. Two of those animals previously had been trained for fMRI experiments. No obvious behavioral changes were observed in any animal following injection. Three objective behavioral measures were available for the fMRI-trained monkeys: 1) the response accuracy to orientation changes in the visual cue, 2) the duration of fMRI experiments as defined by the monkeys' willingness to work for apple juice, and 3) the total percent fixation time during an experiment (in one monkey). In these studies, the correct response ratio following injection of MION (> 98% correct) was similar to results obtained without MION. Monkeys also continued to work as long after MION injection (typically 3-5 hours) as they had without the use of contrast agent. In the monkey in which we directly measured the eye movements (using an MR-compatible eye-track system), we did observe no decrease in fixation performance before and after injection of MION.

Functional Sensitivity

Figure 4 compares representative statistical parametric brain maps generated from single imaging sessions using either the BOLD or CBV method. The image pair shows the same coronal slices through area MT after the acquisition of an equal number of functional volumes (80 volumes). The same statistical threshold of $Z < 3.7$ has been applied to both functional maps. The improved statistical power obtained by the use of contrast agent is obvious in area MT. Taken into account this relatively low number of functional volumes, we obtained only unilateral activation of area MT using BOLD imaging. Of course, as shown in figure 1, bilateral MT activation can be detected after the acquisition of a larger number of functional volumes. By using MION, however, the expected bilateral activation of MT becomes apparent after the acquisition of only 80 functional images. In addition, the bilateral motion selectivity in the intraparietal sulcus reaches statistical significance only in the MION experiments. Moreover, contrast agent improves the anatomical detail in the underlying images. Indeed, the focus within the

figure 4, see page 196

Superior Temporal sulcus is confined to one bank as well as the floor of the sulcus, exactly were we expect to observe motion selective responses. Also in the intraparietal sulcus the activation is restricted to the lateral bank and the fundus of the sulcus. This fits with electrophysiological results showing motion selectivity in areas LIP (in the lateral bank of the IPS) and in area VIP in the fundus of the IPS.

Figure 5 shows average percent signal changes observed for the BOLD and CBV methods in primary visual cortex and MT. Note that the signs of CBV-weighted signal changes and CNR have been reversed for comparison with BOLD results. The use of iron oxide contrast agent increased percent signal changes by a factor of 4 in MT.

Discussion

This study investigated the viability and advantages of employing an iron oxide contrast agent with a long blood half life to enhance functional brain imaging in awake, behaving primates. The primary goal was to improve functional sensitivity for neuroscience applications in this model. We found that the method greatly improved the quality of functional brain maps, and these monkeys showed no obvious adverse effects due to agent injection.

Toxicity

In the three animals tested, no behavioral changes due to injection of agent were observed, even after repeated injections of 10 mg-Fe/kg on several successive days. The iron oxide contrast agent used in this study (MION) is classified as an ultra-small superparamagnetic iron oxide (USPIO), which have shown low toxicity in humans at doses up to 4 mg-Fe/kg. Similar agents currently in clinical trials include AMI-227 (Advanced Magnetics Inc., Cambridge, MA, USA) for imaging liver or splenic lesions (Sharma et al., 1999). The dose required for functional brain imaging is larger than doses selected for clinical studies, because the cerebral blood volume fraction is smaller by a factor of two to four than blood volumes of target organs in the clinical studies. No human data for doses above 4 mg-Fe/kg have been published.

Functional sensitivity

Previous studies in anesthetized rodents using iron oxide contrast agents have shown a large increase in functional sensitivity relative to BOLD signal. This study confirmed these results in the absence of anesthesia and in higher species. Anesthesia could in principle have an effect on functional sensitivity, which can be factored into the product of reactivity and the intrinsic sensitivity per unit change. For BOLD imaging, the intrinsic sensitivity depends upon the baseline concentration of deoxygenated hemoglobin, which in turn depends upon the ratio of oxygen utilization to blood flow. All anesthetics decrease this ratio (Michenfelder, 1988) and thus decrease BOLD sensitivity. Although vascular reactivity to standard perturbations like hypercapnia remains intact, the magnitude of changes may differ from the awake state depending upon the specific choice of anesthesia. Despite these potential differences between awake and anesthetized models, we found that the sensitivity obtained using MION contrast agent was several fold larger than that of BOLD signal in the awake primate.

figures 5, see page 197

Future Possibilities

Future contrast-enhanced studies in this model should improve our understanding of the organization of macaque brain, the relationship between fMRI signals and neuronal activity, the role of CBV in BOLD signal, and whether repeated iron injection produces measurable adverse effects in the non-human primate.

This study employed contrast agent to provide robust signal changes at a relatively coarse isotropic resolution (3 mm). However, the high statistical power of this method could also be used to purchase higher spatial resolution in relation to BOLD studies, where limited sensitivity at low fields is a major reason for the use of large voxel sizes. The improved specificity for parenchymal tissue relative to large vessels (Mandeville and Marota, 1999) provides further advantages in principle for high resolution studies, although the practical advantages of this feature remain to be tested.

An alternative approach for increasing functional sensitivity is to use BOLD imaging at higher magnetic fields, although rodent studies have shown the sensitivity of the CBV-method surpasses that of BOLD signal even at 4.7 Tesla (Mandeville et al., 1998). A disadvantage of higher fields is that macroscopic magnetic field inhomogeneities that arise from susceptibility interfaces (e.g., sinuses or bone) invariably become worse, leading to image distortion and signal dropout. These problems can be combated by shorter gradient echo times or spin echoes; however, those methods reduce BOLD sensitivity at any given field. In contrast, the CBV-weighted method provides an extra degree of freedom, the dose of agent, that can be used to reduce susceptibility artefacts without a loss of sensitivity. As gradient echo times are reduced, or T_2* weighting is removed by the use of spin echoes, more contrast agent can be used to maintain sensitivity. This approach may ultimately be limited by animals' tolerance to the contrast agent for repeated studies.

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- VANDUFFEL W., TOOTELL R.B.H. and ORBAN G.A. Attention-dependent suppression of metabolic activity in the early stages of the macaque visual system. Cereb. Cortex 2000, 10:109-126.

Invited lectures:

- VANDUFFEL W. FMRI in an awake fixating rhesus monkey. Invest. Ophthalmol. Vis. Sci., 1999, 40: VANDUFFEL W. Functional imaging in awake behaving monkeys. PRE-ARVO symposium 2000 (Fort Lauderdale, USA).
- VANDUFFEL W. Functional imaging in awake behaving monkeys. Brain and Cognition symposium. F.C. Donders Center for Cognitive Neuroimaging (Nijmegen, Nederland) October 3, 2000.

Abstracts:

- VANDUFFEL W., BEATSE E., NELISSEN K., TOOTELL R.B.H., TODD J.T. and ORBAN G.A. Areas involved in extracting structure from motion: an fMRI study in the awake fixating monkey. Soc. Neurosci. Abstr., 2000, 26: 304.
- NELISSEN K., VANDUFFEL W., SUNAERT S., JANSSEN P., TOOTELL R.B.H., AND ORBAN G.A. Processing of kinetic boundaries investigated using fMRI and the double-label deoxyglucose technique in awake monkeys. Soc. Neurosci. Abstr., 2000, 26: 304.
- TSAO D.Y., VANDUFFEL W., SASAKY Y., FISHL B., VAN HECKE P., NELISSEN K., ORBAN G.A., AND TOOTELL R.B.H. fMRI of stereopsis in humans and awake behaving monkeys. . Soc. Neurosci. Abstr., 2000, 26: 304.

FIGURES

figure 1

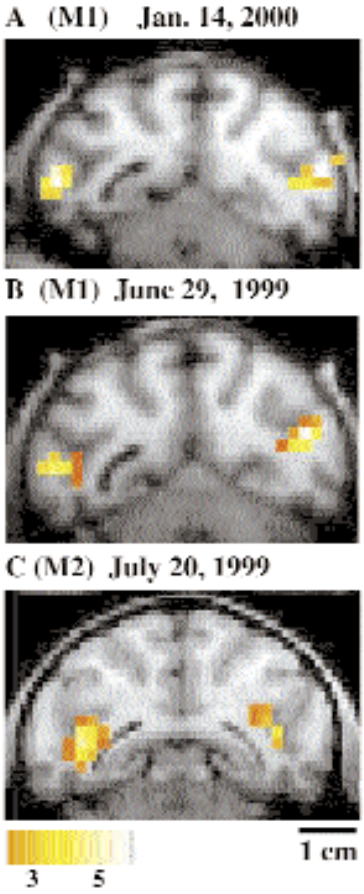

Figure 1: FMRI responses in macaque areas V5/MT+ and V1. Panels A, B and C show bilateral foci of fMRI activity in area V5/MT+, in both monkeys tested (M1, M1 and M2, respectively). The significance was calculated by comparing fMRI responses produced by moving stimuli, minus the activity produced by corresponding stationary stimuli. Only voxels for which the Z-scores exceeded 2.33 are displayed (see color bar below panel B). The activation sites in these coronal slices were located in the fundus and posterior bank of the superior temporal sulcus, exactly where area MT and MST (i.e. area 'V5/MT+') have been localized using previous techniques. The maps in panels A and B were taken from the same monkey, from different scan sessions approximately 6.5 months apart.

figure 2

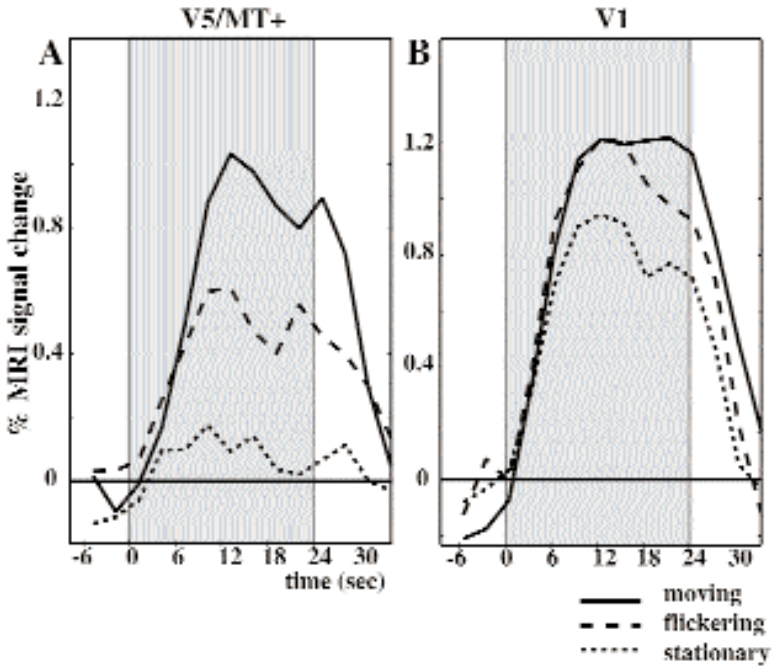

Figure 2: Panels A and B show the time courses of fMRI activity (in most significant voxels) from areas V5/MT+ and V1 (respectively). The averaged time course is shown from 24-second blocks (filled grey) in which the random dots were either moving (solid lines), flickering (dashed lines), or stationary (dotted lines). The filled-white region shows fMRI activity during intervening blocks in which the monkey fixated only a uniformly grey screen (data from 5 scans of M1).

figure 3

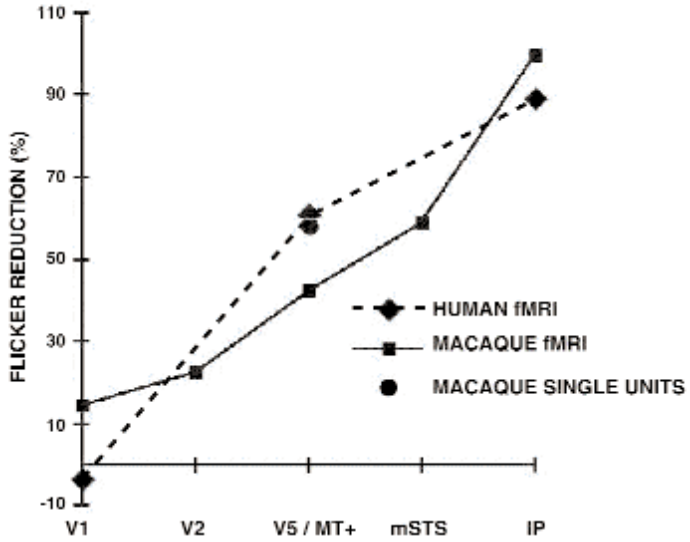

Figure 3: Measurements of flicker reduction suggest a progressive and hierarchical change, across both species (human versus macaque) and across techniques (fMRI versus single units). The filled squares connected by a solid line show the flicker reduction curve based on the present fMRI measurements obtained at high contrast (80%, 8Hz) in 2 macaques. Same data as in Figure 5 are shown, but only for the four motion responsive areas identified with certainty. The triangles connected by a dashed line show the plot obtained in M1 at low contrast (10%, 6Hz). The filled diamonds connected with a stippled line are based on previous measurements from human fMRI (Sunaert et al., 1999), using nearly identical high contrast (95%, 6Hz) stimuli, MR and analysis procedures. The human measurements were based on 3 normal subjects. In retinotopic V1, the human data were sampled from eccentricities comparable to those from which the macaque data were sampled. The filled circle shows the aggregate single unit flicker reduction reported by Qian et al. (1994a), as described in the text. The correspondence between all three measures is fairly good.

figure 4

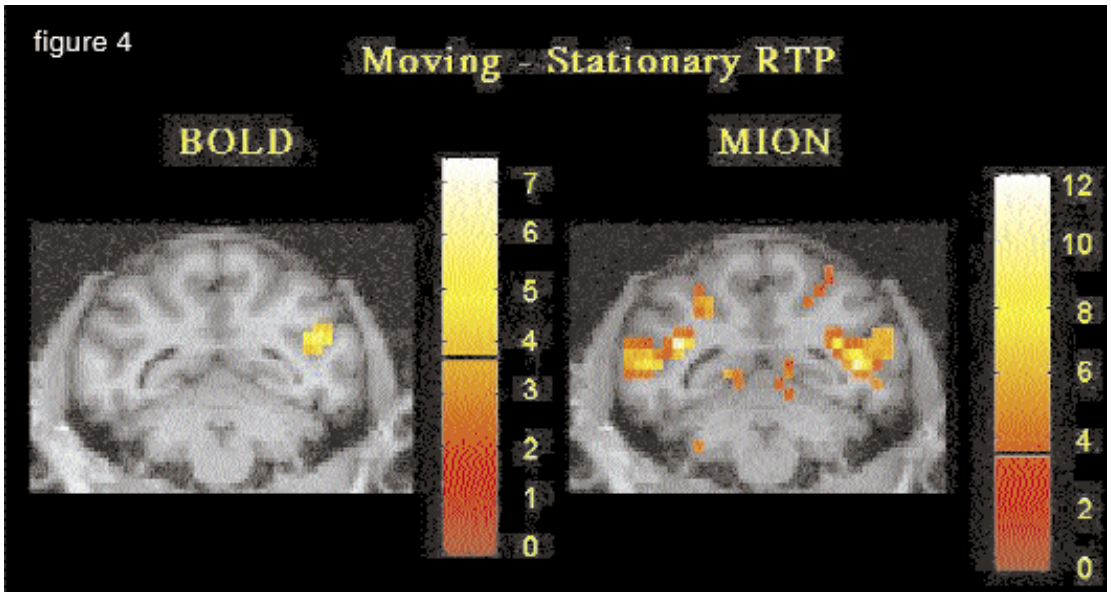

Figure 4: Representative functional brain maps obtained using BOLD signal and CBV-weighted signal. The two images for each method represent a coronal slice through MT. In both maps, the color overlay shows Z-scores above a threshold of 3.7. The use of contrast agent improved both statistical scores and anatomical detail.

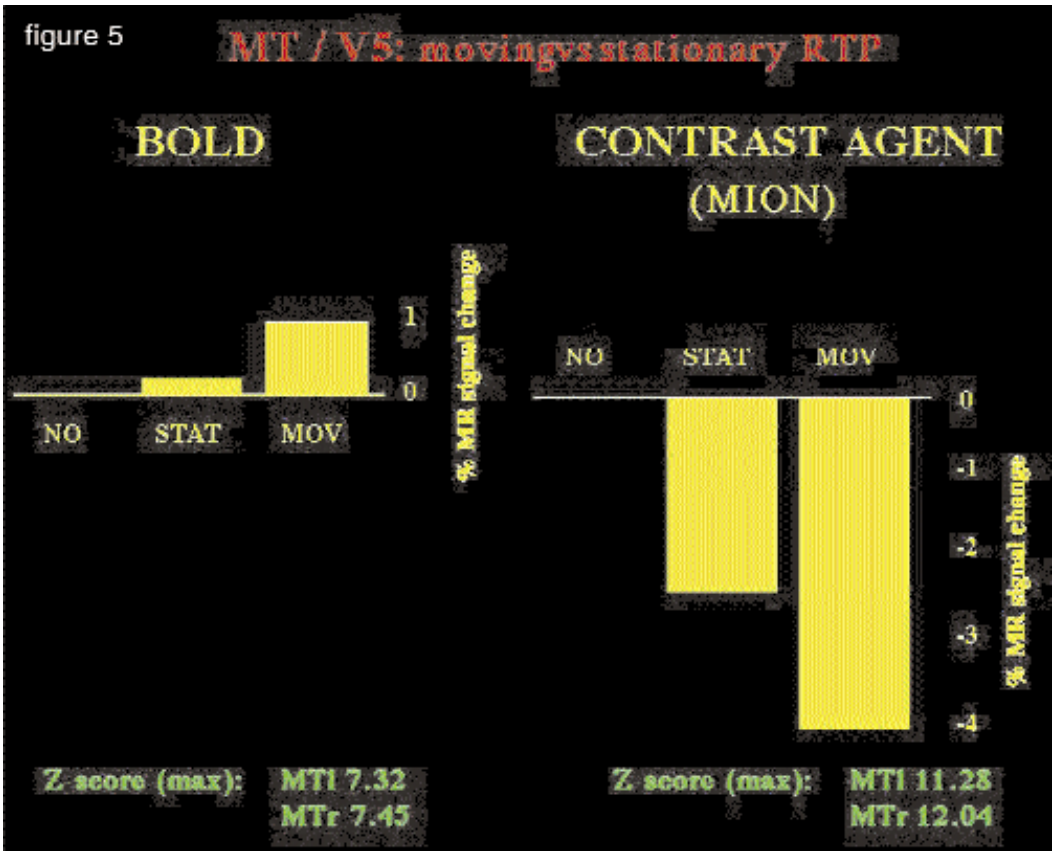

Figure 5: Average percent signal changes in MT using BOLD and CBV-weighted signal. In addition the maximal Z-scores in left and right MTare indicated for both methods.

Report of the Research Group of

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FUNCTIONAL MAGNETIC RESONANCE IMAGING (fMRI) OF THE HUMAN BRAIN

During the past year we pursued our research on various aspects of the visual system in the human, making use of the non-invasive technique of functional magnetic resonance imaging (fMRI) which provides total brain imaging of neuronal activation with an inherent spatial resolution of about 3x3x3 mm and a temporal resolution of the order of a few seconds (cfr. our activity report 1996-98). Some of the projects, all in close cooperation with the group of Prof. G.A. Orban (Neurophysiology, K.U.Leuven), were completed, others were continued or initiated as described below.

The visual processing of motion and task dependency: study of speed identification

S. Sunaert, P. Van Hecke, G. Marchal, G.A. Orban
Attention to speed of motion, speed discrimination, and task difficulty: An fMRI study
NeuroImage 11, 612-623 (2000)

This study, discussed in the 1999 report was completed and the results were recently published in *NeuroImage*.
The work describes the cortical activity in response to attention to speed of motion and the modulation of this response by the level of difficulty of the speed discrimination task.

Extraction of 3D structure from motion

G.A. Orban, S. Sunaert, J.T. Todd, P. Van Hecke, G. Marchal:
Human cortical regions involved in extracting depth from motion
Neuron, 24, 929-940 (1999)

The results of this study, reported last year, were published in Neuron at the very end of 1999. As predicted from monkey data, the results showed that the human homologue of MT/V5 is part of a network of activated regions with right hemisphere dominance, which are involved in extracting depth from motion, including a lateral occipital region, five sites along the intraparietal sulcus (IPS), and two ventral occipital regions.

Further research is now being pursued on the effects of stimulus type (random lines and dot surfaces) and of transparency on the extraction of 3D structure from motion.

3D shape cues interact differently in dorsal and ventral parts of the human visual system

S. Sunaert, J.T. Todd, P. Van Hecke, G.A. Orban

The previous study (item 2) demonstrated that the visual cortex of humans contains several regions involved in the processing of 3D structure from motion. The present study investigates 1) the effect of static pictorial depth cues on those regions involved in extracting structure from motion, and 2) the interaction of different cues in these regions.

Psychophysical studies have shown that if the structures suggested by the different cues are similar, the 3D shape is a weighted average of the various sources of information, while if the

cues are discordant, the perception of the 3D shape will rely on the most salient one.

Our fMRI study performed on 8 subjects used a 2x2 factorial design with pictorial and motion 3D shape cues as factors, and consisted of the following 6 conditions (stimuli similar to those of the previous study): 3D polyhedra rotating in depth (3D motion), resp. translating in the fronto-parallel plane (2D motion), patterns of 2D random lines rotating (3D motion), resp. translating (2D motion), stationary views of 3D polyhedra, resp. of 2D random line patterns (the latter 2 conditions to assess the effects of pictorial cues in the absence of motion).

Although the *pictorial* cues had little effect of their own, they interacted strongly with the processing of 3D structure from *motion*. More interestingly, the two cues were found to interact differently in different parts of the visual system. Static pictorial information enhances the activation related to the processing of 3D shape from motion in ventral regions left collateral sulcus), whereas it attenuates this activation in dorsal regions (right and left hMT/V5+, right middle occipital gyrus and lateral occipital gyrus). These effects occur only if the pictorial and the motion cues are in agreement with one another. This was demonstrated in a second experiment in which rectangular polyhedrons were presented that had been stretched in depth so that the angle between adjacent faces was no longer 90 degrees. When viewed from the initial appropriate orientation, these objects appeared as untransformed rectangular polyhedra, but once they rotate their distortion appearing from the structure from motion conflicts with the initial information from the static pictorial cue. In all the above areas, both dorsal and ventral, the cue conflict displays produced activation patterns comparable to those for the 3D rotations of random lines, showing no significant cue related increase or decrease in activity.

The findings of this study will be submitted for publication early next year.

Orientation discrimination of gratings and objects

I. Faillenot, S. Sunaert, P. Van Hecke and G.A. Orban
Eur. J. Neuroscience, in press

The results of this study which was reported last year are now being published in European Journal of Neuroscience.
The main conclusion of the study was that, in contradiction to to generally accepted views, orientation discrimination of gratings and objects involve largely similar networks in both ventral and dorsal visual regions.

Attention mechanisms in visual search – an fMRI study

U. Leonards, S. Sunaert, P. Van Hecke, G.A. Orban:
J. Cogn. NeuroScience, 12: Suppl 2, 61-75 (2000)

The human visual system is usually confronted with many different objects at a time. While some objects attract our attention instantaneously, we often need to scrutinize a scene to find a particular object. Psychophysical reaction-time studies have identified two different strategies by which objects can be found: an automatic , efficient search process in which the object pops out of the scene, and a higher-level conscious, so-called inefficient search, depending on the number of distractors and the type of target. Two different theories have been proposed to

explain these search processes. Parallel theories assume that both types of search are regulated by a single mechanism that is modulated by attentional and computational demands. Serial theories, on the other hand, suggest that parallel processing may underlie efficient search, but that inefficient search requires an additional serial mechanism, an attentional "spotlight" that successfully shifts attention to different locations in the visual field.

fMRI was used in this study on 12 volunteers, to investigate the neural basis of efficient and inefficient visual search,. While maintaining fixation, the subjects searched for targets that differed from surrounding distracters either by orientation or luminance (feature visual search (FVS)), or by conjunction of orientation and contrast polarity (conjunction visual search (CVS)). Feature search invoked a search pattern in which reaction times did not increase with the number of distracters, corresponding to an efficient search pattern. During conjunction search, reaction times increased with the number of distractors and were longer for target-absent than for target-present conditions, corresponding to an inefficient search pattern.

Analysis of the fMRI results shows that the cerebral networks involved in efficient and inefficient search overlap almost completely (regions along the intrapaietal sulcus and several extrastriate regions). Only the superior frontal region, known to be involved in working memory, was specifically involved in inefficient search (but this region was shown to be distinct from the frontal eye field controlling spatial shifts of attention). Activation intensity correlated most strongly with the subjects' search process in the extrastriate cortical areas, where the amount of activation depended on the number of distractors in the display. Such correlation however was not observed in the parietal and frontal regions, usually assumed to as being equally involved in spatial attention processing. These findings support the object search models based on parallel processing, unless we assume the existence of an oculo-motor independent high-speed serial searchlight, operating in the extrastriate cortex.

Event-related fMRI

E. Béatse, S. Sunaert, P. Van Hecke, G.A. Orban

Event-related fMRI records the neuronal response after a short stimulus (event). This recent and very promising technique gets rid of undesired or physiologically unrealistic effects such as habituation, predictability, fatigue and so on, inherent to continuous or blocked tasks used in conventional fMRI and PET imaging., After our initial efforts to set up the appropriate acquisition technique and processing methods (see previous report), we initiated a first event-related fMRI study of the visual system in the human and choose to take up our previous study on speed discrimination (see item 1 above) in order to compare results using both techniques and identify common activations and possible new regions when subjects have to perform the discrimination task after presentation of single randomly selected speed differences, rather than during blocks of constant speed differences.

The stimulus was a random-dot-pattern (4° diameter) which was stationary (reference) or moving linearly to the right during a time between 1.5 and 2.5 s (= «event»). Subjects had to decide whether the speed was smaller or larger than the reference speed of 6°/s (right/left key press). Four subjects were trained to discriminate speed at 5 different levels of speed difference until their performance scores were stable. The stimuli were presented in pseudo-random order

of difficulty. The subject had thus no clue on the speed of the next stimulus. fMRI signals were processed using the SPM99 event-related statistical analysis, in which the response to an event is modelled as a convolution of the impulse event function and the hemodynamic response function. Various forms for the latter function were implemented (standard, standard and derivative, gamma functions). Results were evaluated using constrasts between the various event types.

Comparison of speed identification (independent of difficulty level) vs. stationary, yielded the same pattern of activation as in our study on speed identification using a block design (Sunaert et al., see item 1 above), validating the reliability of the event-related technique. Also, the linear increase of the activation as a function of the difficulty level, was found for the same areas (as well as for a few more) as in the block design, and was moreover much stronger and significant.

The completion of this study is being delayed due the departure of the responsible researcher (E.B), end of August 2000.

PUBLICATIONS

- G.A. Orban, S. Sunaert, J.T. Todd, P. Van Hecke, G. Marchal:
  Human cortical regions involved in extracting depth from motion
  Neuron, 24, 929-940 (1999)
- S. Sunaert, P. Van Hecke, G. Marchal, G.A. Orban
  Attention to speed of motion, speed discrimination, and task difficulty: An fMRI study
  NeuroImage 11, 612-623 (2000)
- U. Leonards, S. Sunaert, P. Van Hecke, G.A. Orban:
  Attention mechanisms in visual search – an fMRI study
  J. Cogn. NeuroScience 12: Suppl. 2, 61-75 (2000)
- I. Faillenot, S. Sunaert, P. Van Hecke and G.A. Orban :
  Orientation discrimination of gratings and objects compared : an fMRI study
  Eur. J. Neuroscience (2000), in press

ABSTRACTS

- S. Sunaert, J. Todd, P. Van Hecke, G. Marchal, G.A. Orban:
  Depth from perspective modulates the human cortical regions involved in extracting depth from motion
  NeuroImage, 11, Pt2, S731 (2000)
  Sixth Annual Meeting of the Organization for Human Brain Mapping, San Antonio, June 2000
- G.A. Orban, I. Faillenot, P. Van Hecke
  Attention to global patterns involves fusiform and prefrontal cortex in the right hemisphere
  Society for Neuroscience, Abstracts, vol. 30, Part 1, 106.6 (30th Annual Meeting, New Orleans, November 2000)
- S. Sunaert, G. Fesl, J.Todd, A. Rosier, P. Van Hecke, G.A. Orban
  Human cortical regions involved in extracting 3D structure from motion depend on stimulus type and transparency
  Society for Neuroscience, Abstracts, vol. 30, Part 2, 593.10 (30th Annual Meeting, New Orleans, November 2000)

Report of the Research Group of

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Investigation of the molecular mechanisms underlying reorganization of cortical topography after limited sensory deafferentation

Introduction

The adult mammalian brain is capable to reorganize dramatically in answer to large-scale peripheral deafferentations. The loss of sensory input due to restricted deafferentations or injury to the sensory surface results in massive changes in the sensory representations. This reorganization typically involves shifts in the topography of the functional representations. The part of cortex that normally represented the lesioned portion of the sensory surface will become occupied by the representation of adjacent sensory surface.

A common mechanism for different forms of adult plasticity is the potentiation of previously existing sub-threshold inputs through local changes in synaptic weight. In addition, the CNS has the capacity for axonal and dendritic growth and synapse turnover in order to adapt the circuitry. New synaptic contacts would become the new source of activation for sensory-deprived neurons. Both phenomena could occur at the cortical and subcortical level in order to contribute to the cortical reorganization.

In the somatosensory system compelling evidence exists for a distinct subcortical contribution to the cortical reorganization following sensory denervations. However, in the visual system, electrophysiological experiments and tracing studies did not reveal a significant contribution of the geniculate nucleus to cortical reorganization after retinal lesions. Therefore in the visual system the long-range horizontal connections are recognized as the prime candidates for effecting reorganization in visual cortex.

The molecular components involved in cortical reorganization remain largely unknown. Several immediate early genes, amino acid neurotransmitters, growth factors and neurotrophins have already been implicated in adult brain plasticity. Nevertheless, the molecular cascade responsible for representational plasticity remains illusive. The main goal of our research is to detect new molecules with a role in cortical plasticity using the visual system of retinal lesion cats as working model.

As before our investigations of the past year were mainly foccussed on the search for molecular differences between reorganizing and normal visual cortex.

Previous investigations with the DDRT-PCR method resulted in the detection of several genes with a differential expression between visually-deprived and normal cortex. We therefore introduced complementary RNA-based methods to confirm our DDRT-PCR findings for the known genes Cu/Zn Superoxide dismutase 1 (SOD1), Myocyte-specific enhancer factor 2A (MEF2A), cyclophilin A and thymosin beta4. We also used western blotting and immunocytochemistry to extend our data to the protein level.

Since previous immunocytochemical and biochemical investigations of the excitatory neurotransmitter glutamate revealed a function for this transmitter in cortical plasticity (Arckens et al., 2000) we started the investigation of glutamate receptors and transporters in the visual cortex of normal cats and animals with retinal lesions. Glutamate receptors take part in changes con-

cerning the efficiency of synaptic transmission thought to underly not only adult cortical plasticity but also learning, memory and the formation of neuronal connections during development. Glutamate transporters are important for the clearance of glutamate from the synaptic cleft to avoid the accumulation of high, neurotoxic glutamate levels in the brain. Furthermore, we used microdialysis to investigate possible differences in the release of glutamate from normal and deprived visual cortex.

Differences in protein expression are not always related to differences in mRNA expression and posttranslational modifications may also fullfill an important function in brain plasticity. We therefore used functional proteomics and phosphorylation studies to investigate such differences in the visual cortex of retinal lesion cats.

Results

Cyclophilin A

DDRT-PCR revealed decreased cyclophilin A mRNA expression in lesion-sensitive cortex 14 days after the induction of retinal lesions compared to normal visual cortex. As reported last year we were able to confirm these findings with *in situ* hybridisation and competitive PCR. Furthermore immunocytochemistry extended these findings to the protein level. Despite the many efforts to develop a quick and reliable screening method to analyze the many candidate gene-fragments isolated in DDRT-PCR, non of the tested techniques, including northern, reverse northern and dot blotting, gave satisfactory results. Based on the fact that the differences in gene expression as detected with DDRT-PCR are only physiologically meaningful when translated into differences in protein expression we have introduced semi-quantitative western blotting as a possible screening method. We developed this method first for cyclophilin A because for this gene we had already gathered convincing evidence for differential gene and protein expression. Indeed western blotting confirmed our immunocytochemical findings in showing decreased cyclophilin A protein levels in lesion-affected cortex compared to normal visual cortex. In the context of the analysis of other candidate-genes with a role in cortical plasticity this technique was found to fullfil a double role. Western blotting can extend DDRT-PCR results to the protein level and can be established in an acceptable amount of time to be applicable to a large number of genes. The only drawback is the need for specific antibodies against the protein under investigation.

SOD1, MEF2A

Differential display on total RNA of a retinal lesion cat three days after the induction of retinal lesions revealed the differential expression of two known genes, Cu, Zn superoxide dismutase (SOD1) and myocyte specific enhancer factor-2A (MEF2A) among a series of unknown genes. We confirmed the differential expression of SOD1 and MEF2A using semi-quantitative RT-PCR. Based on our findings for cyclophilin A we have used western blotting to confirm and extend these data to the protein level.

SOD1 is an antioxidant enzyme that protects cells from oxidative damage by dismutating the superoxide ion to hydrogen peroxide. Both differential display and semi-quantitative RT-PCR indicated that SOD1 mRNA shows a significantly ($p<0.05$) higher expression in deprived (cen-

tral) cortex compared to non-deprived (peripheral) cortex of retinal lesion cats. This difference was not observed in control animals.

Western blotting revealed the differential expression of SOD1 protein. Again there was a higher concentration of SOD1 in central (deprived) cortex of retinal lesion cats whereas in control animals more SOD1 protein was present in peripheral cortex.

This differential expression could be a consequence of topographic reorganization but could also be due to the loss of visual stimulation. To exclude this possibility, semi-quantitative PCR was performed on total RNA from isolated hemisphere cats. The left hemisphere of these animals was deprived of all visual input by cutting the left optic tract and the corpus callosum. If there is a difference in SOD1 expression between the hemispheres of these cats it can only be due to the loss of visual input since no topographic reorganization takes place in these animals due to lack of nearby normal visual cortex in the lesion-affected hemisphere. Indeed, we observed no difference in SOD1 mRNA expression between the left and the right hemisphere of an isolated hemisphere cat thereby implicating SOD1 in topographic map reorganization in adult cat visual cortex in answer to a partial loss of sensory stimulation.

MEF-2A is a transcription factor belonging to the MADS-box family. It is involved in the differentiation process of both muscle and brain. Both differential display and semi-quantitative RT-PCR revealed significantly ($p<0.05$) higher MEF-2A expression in sensory-deprived cortex of retinal lesion cats compared to its normal counterparts. For control subjects the reverse was true : they showed higher MEF-2A mRNA concentrations in peripheral cortex ($p<0.05$).

Thymosin beta-4

Although differential display implicated thymosin beta-4 in cortical plasticity, we were unable to confirm these findings in *in situ* hybridisation. Also western blotting did not support the display data. However both *in situ* hybridization and immunocytochemistry revealed that changes in thymosin mRNA expression levels may be linked to gliosis. We therefore started immunocytochemical double stainings to determine the cell type expressing thymosin beta-4 in cat visual cortex. Both neurons and glia wer found to be immunopositive. To determine what glial cells are involved we performed double stainings between thymosin beta-4 and specific markers for astrocytes (GFAP), oligodendrocytes (O4, CNPase, Gal-C, anti-oligodendrocyte) and microglia (CD11b, OX-42). Based on these findings we can exclude astrocytes and oligodendrocytes indicating that microglia is the responsible celltype for glial thymosin staining. However up till now we were unable to find antibodies that label specifically the microglial cells in cat brain. We are currently investigating the possibility to identify the thymosin beta-4 positive glial cells as microglial cells by labeling them histochemically with isolectin B4.

In the context of this investigation of the glial cells in cat visual cortex it became clear that with one particular antibody, anti-oligodendrocyte, we were able to delineate the different visual areas in cat cortex. Since the antibody was raised against a homogenate of rat glial membranes and white matter, we believe that it might recognize myelin. The typical staining pattern for each visual area might therefore be linked to differences in myelination. We are currently analysing the visual cortex of monkey. Preliminary data indicate that also in monkey different visual areas are characterized by a different staining of immunoreactive fibers with this antibody.

Development of a sensitive RT-competitive PCR method for studying Immediate early gene mRNA expression in cat visual system.

We developed a PCR method to analyze and quantitate the effect of binocular central retinal lesions on the mRNA expression level of two Immediate early genes (IEGs), c-fos and zif-268. Earlier studies using *in situ* hybridization and immunocytochemistry already showed that the expression of these genes in visual cortex was altered after retinal lesions

The combined use of reverse transcription followed by the polymerase chain reaction makes it possible to detect small amounts of mRNA. However, this conventional RT-PCR method is not quantitative. We employed a specific competitive PCR method to quantitate cat gene expression levels. Recently we cloned a fragment of cat c-fos (357 bp) and zif-268 gene (381 bp) and determined the nucleotide sequence. For RT-cPCR, competitor DNA fragments (mimic) were generated containing the same primer template as cat c-fos and zif-268 DNA fragment (target), but with a completely different intervening sequence. As the primers are identical, target and mimic compete for primer binding and amplification, and are amplified with the same efficiency. Serial dilutions of the mimic fragment were added to the PCR reactions containing a constant amount of cat DNA. PCR products were electrophoresed and the amount of target DNA was determined from the amount of competitor DNA at which the densities of target and competitor bands were equal in densitometric analysis. Furthermore, the densitometric analysis of the bands corresponding to the target and the mimic were further used to construct a standard curve from which a more accurate amount of target DNA was derived. Sample to sample variations in the quantity and quality of starting total RNA was considerably attenuated by normalizing the results to GAPDH mRNA expression used as a housekeeping gene.

From our results, it is shown that there is a significant decrease in c-fos and zif-268 mRNA expression within the lesion-affected cortex if compared to the normal peripheral cortex of adult cats. No significant decrease could be detected in control cats (without a retinal lesion). The mRNA expression level for both IEGs, c-fos and zif-268, within the lesion-sensitive area is examined for five cats and measures on average 50% compared to the respectively mRNA expression levels within the peripheral cortex.

Microdialysis for glutamate: analysis of extracellular concentrations

Preceeding microdialysis experiments already showed a difference in extracellular glutamate and aspartate concentrations between normal and sensory deprived visual cortex of cats with retinal lesions. In contrast, sampling from visual cortex of normal animals revealed similar amino acid concentrations over the whole visual cortex.

In new experiments we chose to analyse the effect of perfusing the microdialysis probes with artificial cerebrospinal fluid containing 4mM PDC, after a stable baseline in aspartate and glutamate concentrations was reached. PDC (L-trans-pyrrolidine-3,4-dicarboxylic acid) is one of the most potent inhibitors of glutamate transport. Results of microdialysis experiments in normal animals showed that PDC perfusion results in an immediate increase in extracellular aspartate and glutamate concentrations. This finding was very constant over different probes and experiments.

By comparing the percentual increase in concentration of both neurotransmitters between normal and sensory deprived cortex of lesioned animals, we will be able to decide whether the difference in concentrations of extracellular glutamate and aspartate between both regions is a consequence of a difference in re-uptake back into glia and postsynaptic terminals. Also, in the future we will combine perfusion with 4mM PDC with perfusion of 1mM DNQX (6,7-dinitroquinoxaline-2,3-dione), an antagonist of the NMDA receptor in order to investigate a possible role for this type of glutamate receptor in cortical plasticity.

Glutamate receptors

There are two major classes of glutamate receptors: the G-protein coupled or metabotropic glutamate receptors and the glutamate-regulated ionchannels or ionotropic glutamate receptors. Three types of ionotropic glutamate receptors are described, the AMPA, NMDA and kainate receptors, all named after their agonist. AMPA and NMDA receptors are mediating the larger part of excitatory synaptic transmission in the brain. In human, four AMPA receptor subunits (GluR1-4) and five NMDA receptor subunits (NMDA1 & NMDA2A-D) have been described.

We are particularly interested in how the AMPA and NMDA receptors in area 17 of the visual cortex in adult cat will react during cortical reorganization after the induction of the retinal lesion. The sequences of the different subunits are not known in cat. For each receptor subunit, we have chosen up- and downstream primers based on conserved regions in the known sequences of human and rat. All primers were chosen at the 3'-terminal region of each subunit. With these primers, we could amplify a part of the cat-specific sequence in RT-PCR. The amplified cDNA-fragments were cloned into a pCR™ II vector. Using the dideoxy method of Sanger, we have sequenced the cDNA-fragments obtained by RT-PCR. Until now, we know part of the nucleotide sequence at the 3'-terminal region of the four AMPA receptor subunits (GluR1-4) and the five NMDA receptor subunits (NMDA1 & NMDA2A-D) in cat. All the obtained cat-specific sequences are more homologous to human- (from 89% to 99% homology) than to rat-specific sequence, except for GluR2 that has a sequence-homology of 94% with the rat sequence. Based on these cat-specific sequences, we are able to synthesize cat-specific probes for *in situ* hybridization. With this technique, we want to investigate if there is a differential expression of the glutamate receptors on the level of transcription in deprived versus non-deprived cortex. We are also interested in possible changes on the protein level. Therefore, we use subunit-specific polycloncal antibodies (Chemicon) in Western Blotting and immunocytochemical studies. Using Western Blotting, we have found that in normal cats there is a higher expression of the GluR1-subunit in the central region of area 17 compared to the peripheral region, but in retinal lesion cats (2 weeks post-lesion) the expression of GluR1 is higher in the peripheral, normal cortex than in the visually deprived cortex. We are continuing our experiments for the other subunits.

Glutamate transporters

In an attempt to detect differences in expression of glutamate transporters between normal and sensory deprived visual cortex following retinal lesions, we started the analysis of the expression of the four known glutamate transporters (EAAT1-4) expressed in brain at the mRNA

level. Hereto, sequences of cat specific cDNA fragments of the 4 transporters were determined. This information enabled us to develop a competitive PCR and to perform *in situ* hybridization with cat-specific oligonucleotides specific for each of the four transporters. The first results indicated that glutamate transporter mRNA was not differentially expressed between normal and sensory-deprived visual cortex. For this reason we decided to change our field of interest to the protein level.

Antibodies against EAAT1, 2 and 4 were geneourously provided by other research groups and enabled us to perform Western blotting and immunocytochemistry. Antibodies against EAAT3 were developed by immunizing rabbits with a synthetic peptide specific for cat EAAT3. For all these antibodies the specificity was determined in Western blotting experiments with cerebellar tissue as postive control. Each antibody labeled bands of expected size in cerebellum and visual cortex. Immunocytochemical staining of visual cortex of normal cat with these same antibodies resulted in neuropil staining for EAAT1 and 2 and the staining of neurons for EAAT3 and 4. In future experiments sections of visual cortex of retinal lesion cats will be stained and semi-quantitative Western blotting will be used to determine possible differences in the expression of glutamate transporter proteins between normal and sensory deprived visual cortex.

Our findings for EAAT4 in visual cortex of normal animals were unexpected. EAAT4 is commonly known as a glutamate transporter which is confined to the Purkinje cells of the cerebellum. Surprisingly, our experiments revealed EAAT4 expression in the cerebral cortex of the cat both at the mRNA and the protein level. Further experiments in which we compared the cerebral and cerebellar cortex of both cat and mouse enabled us to proove that EAAT4 is indeed expressed in the cerebral cortex, predominantly in neurons of layers II/III and V with a somatodendritic localisation indicating that these results for EAAT4 can be generalized to all mammals. (These results will appear in Neuroreport, february 2001)

Posttranslational modifications: the analysis of protein phosporylation

The physiological activities of enzymes and structural proteins within the cell are often regulated by changes in their post-translational modifications (phosphorylations, glycosylations,…). Consequently, the retinotopic reorganization, occuring in adult cat sensory deprived visual cortex, which is thought to rely on changes in protein expression, may as well be accompanied by changes at the levels of these post-translational modifications. To investigate these changes, we optimized a method for the specific detection of the phosphorylation state of the proteins present in the visual cortex of the adult cat, based on two-dimensional electrophoresis and western blotting. Using antibodies specifically recognizing phosphorylated tyrosine residues we are able to visualize proteins possessing such a modified amino acid.

Proteins with an iso-electric point between pH 4 and pH 7 were separated by two-dimensional electrophoresis and blotted to a PVDF-membrane. Comparison of the tyrosine phosphorylation levels of the proteins in the deprived and the non-deprived parts of the primary visual cortex of retinal lesion cats showed no differences 14 days post-lesion, the time point chosen for this study. Neither were differences observed between normal cats and retinal lesion cats.

In order to completely understand the physiological importance of a phosphorylated protein, it is not only necessary to know *when* that protein is modified. The exact position of the

altered amino acid residue is equally important as some positions have a larger impact on the activity of the protein than others.

To analyse the positions of these modifications, we optimized a method using some recently developed proteomics tools. First of all, the phosphorylated protein is proteolytically digested using trypsin. From the resulting tryptic peptides, the phosphorylated peptides are specifically enriched by metal affinity chromatography in a microtip, based on the complexation of phosphate (and phosphopeptides) by some metals, for example Ga^{3+} and Fe^{3+}. The masses of these phosphopeptides are then measured on a matrix assisted laser desorption/ionisation time-of-flight mass spectrometer (MALDI-TOF-MS). After comparison of these masses with the theoretical tryptic digest of the protein, this makes it possible to identify the phosphorylation sites.

So far, this method enabled us to identify the phosphorylation sites of the bovine alpha- and beta-casein.

Neurofilament protein expression : a selective marker for cat visual areas

A monoclonal antibody (SMI-32, Sternberger Monoclonals Inc.) that recognizes a non-phosphorylated epitope on the medium (168 kD) and high (200 kD) molecular-weight subunits of neurofilament proteins, has been used in an immunocytochemical study of cat brain. This antibody labels primarily the cell body and apical dentrites of a particular subset of pyramidal neurons, especially in supra- and infragranular layers. In addition, there was a dense neuropil staining in the corresponding layers. Layers I and IV were devoid of immunoreactive neurons. Layer V contained the most intensely labeled and largest neurons with an extensive dendritic pattern: the apical dendrite towards layer III was conspicuous and the smaller horizontal spreading dendrites were also darkly stained.

Neurofilament protein-immunoreactive neurons were prominent in seventeen visual cortical areas (areas 17, 18, 19, 20a, 20b, 21a, 21b, 7, posteromedial lateral, posterolateral lateral, anteromedial lateral, anterolateral lateral, dorsal lateral, ventral lateral and posterior suprasylvian areas and splenial and cingulate visual areas). We examined carefully the staining patterns of all these areas. This monoclonal antibody against neurofilament protein revealed a characteristic pattern of immunostaining in each area: size, shape and density of neurofilament protein-immunoreactive neurons differed substantially across all cortical areas. Moreover, it was also obvious that several visual areas showed differences in laminar distribution and that such profiles may be used to delineate various cortical areas in cat visual system. Therefore, the expression of neurofilament protein can be use d as a specific marker for the precise localization of the boundaries between cortical areas. These features provide considerable utility for comparison with the cortical maps from Tusa et al. (1981) and cat brain atlas. We have also found strong immunopositive cells in the dorsal part of the lateral geniculate nucleus (dLGN) and in the medial interlaminar nucleus (MIN).

POMC

Pro-opiomelanocortin (POMC) is the polypeptide precursor of N-terminal POMC, adrenocorticotropic hormone (ACTH), α-, β- and γ-melanotropin (α-, β-, γ-MSH), the opioid hormone

β-endorphine (β-end) and β-lipotropin (β-LPH). The aim of the present project is to study the effects of different stress types (processive vs. systemic and acute vs. chronic stressors) on the POMC-processing in the chicken.

The goal of the present study was to develop a competitive PCR assay to measure changes in the expression of pro-opiomelanocortin (POMC) mRNA in myelomonocytic HD11 cells after *Salmonella typhimurium* lipopolysaccharide (LPS) stimulation. HD11 cells are chicken myelomonocytic cells (macrophages) transformed by the *v-myc*-encoding MC29 virus. Pro-opiomelanocortin mRNA could be detected in HD11 cells by reverse transcriptase – polymerase chain reaction (RT-PCR). To our knowledge, this is the first observation of a POMC RNA transcript in avian macrophages. Based on this observation, the effect of stimulation with bacterial LPS on the POMC expression in HD11 cells was investigated by means of a newly developed competitive PCR assay. For this purpose, the HD11 cells received a one-hour LPS challenge with LPS concentrations ranging from 0 to 100 ng/ml. A PCR MIMIC (consisting of a heterologous DNA fragment flanked by templates for the gene-specific primers) was used as an internal control in the competitive PCR. A ten-fold dilution series of the MIMIC was co-amplified with a constant amount of experimental cDNA. While HD11 POMC mRNA expression showed an increase of one order of magnitude following treatment with 100 ng/ml LPS as compared to untreated controls, no significant differences could be observed after treatment with 50 and 10 ng/ml LPS. Quantitative measurement of POMC mRNA levels is a first step towards a better understanding of the physiological role of non-hypophysial POMC-derived peptides in the response to immune stress in birds.

PACAP and the PACAP Type I receptor in chicken brain

To map in detail the brain areas in which pituitary adenylate cyclase-activating polypepide (PACAP) may play a significant role in birds, the distribution of PACAP and PACAP type I receptor (PAC1-R) mRNA was examined throughout the entire chicken brain by using *in situ* hybridization histochemistry. Widespread distribution of both PACAP and its receptor mRNA were found. The telencephalic areas where the most intense signals for PACAP mRNA were found included the hyperstriatum accessorium, the hippocampus, and the archistriatum. In the diencephalon, a group of neurons that highly expressed PACAP mRNA was observed from the anterior medial hypothalamic nucleus to the inferior hypothalamic nucleus. Moderate expression was found in the paraventricular nucleus and the preoptic region. A second large group of neurons containing PACAP message was found within the nucleus dorsolateralis anterior thalami and extended caudally to the area around the nucleus ovoidalis and the nucleus paramedianus internus thalami. Furthermore, expression of PACAP message was observed within the bed nucleus of the pallial commissure, nucleus spiriformis medialis, optic tectum, cerebellar cortex, olfactory bulbs, and several nuclei within the brainstem (dorsal vagal and parabrachial complex, reticular formation). The highest expression of PAC1-R mRNA was found in the dorsal telencephalon, olfactory bulbs, lateral septum, optc tectum, cerebellum, and throughout the hypothalamus and the thalamus. The presence of PACAP and PAC1-R mRNA in a variety of brain areas in birds suggests that PACAP mediates several physiologically important processes in addition to regulating activity of the pituitary gland.

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1. Coding of 3D-shape in macaque inferior temporal cortex

Real world objects are three-dimensional. Yet, little is know about the coding of the depth structure of objects by visual cortical neurons. It is well known that the inferior temporal (IT) cortex is involved in object recognition. Indeed, single cell studies have shown that IT neurons code object attributes such as 2D contours, texture and color. Recently, we have shown that IT neurons are also selective for the three-dimensional (3D) shape of objects (Janssen et al., PNAS, 1999). We tested for such selectivity by comparing responses to stereo-defined, curved 3D shapes derived from identical pairs of monocular images. More than one third of macaque IT neurons were selective for 3D shape. In the vast majority of those neurons, this selectivity depended on the global binocular disparity gradient and not on the local disparity. Thus, IT cortex processes not only 2D but also 3D-shape information.

The anterior part of the macaque inferior temporal cortex, area TE, occupies a large portion of the temporal lobe. Anatomically, TE can be divided into a number of subregions. Before our studies, no relationship between the anatomical subdivisions and neuronal selectivity had been described. We have provided the first evidence for a functional specialization within TE. Neurons selective for three-dimensional (3D) shape defined by binocular disparity were concentrated in the lower bank of the Superior Temporal Sulcus (STS), whereas neurons in lateral TE were generally not selective for 3D shape, though equally selective for two-dimensional shape. These findings, combined with earlier anatomical results, reveal that TE consists of at least two distinct areas, one of which processes a specific object property. We have published this important result in *Science*.

We have also investigated in more detail how TE neurons represent disparity-defined 3D structure. The selectivity for 3D shape generally consisted of a selectivity for second-order disparity variations, i. e. for changes in the gradient of disparity (as in curved surfaces), which proved to be remarkably sensitive to disparity discontinuities, as in sharp edges and steps in disparity. The majority of the neurons remained selective for small differences in 3D structure. Moreover, 3D-shape selectivity could be preserved when changing the position or the size of the stimulus, which replicates the size- and position invariance for 2D shape. Thus, the representation of 3D shape involves a complex neuronal machinery, consisting of first- and second-order disparity selective neurons, which are remarkably sensitive to small spatial variations of disparity and preserve their selectivity over transformations in size and position. These results have been published in *Neuron*.

In the above experiments, the shapes were filled with a random-dot pattern, and both the boundary of the shape and its surface were curved in depth. The selectivity for 3D shape of TEs neurons could therefore depend on either the disparity variation on the boundary, or the disparity variation on the surface of the shape, or on both. To determine the relative contribution of surface and boundary to the 3D shape-selectivity, we recorded the responses of single neurons to concave and convex 3D shapes, in which either the boundary was curved in depth or the surface, or both. Two stimuli contained only surface disparities: in the restricted 3D surface, the disparity was maximal in the center of the shape and smoothly approached zero towards the boundary, whereas in the large surface, the 3D structure in the center was identical to the restricted surface but the pattern extended beyond this area covering a 13x13 deg square. Two stimuli contained only boundary disparities: in the decorrelation stimulus, the dis-

parity information on the surface of the shape was eliminated by decorrelating the dots between left and right eye, leaving only disparity on its boundary along the vertical axis. In the second boundary disparity stimulus the disparity information on the surface was eliminated by filling the shape with an uniform, texture-less white color (solid shape; cfr. a folded sheet of white paper). After a preliminary test with monocular presentations and a position-in-depth test demonstrating 3D shape-selectivity, 46 neurons were tested in detail. Seventy-two percent of the neurons tested (33/46) were selective for the 3D surfaces (restricted surface: N=29, large surface: N=15), whereas 37% of the neurons tested (17/46, corresponding to 68% of the neurons selective for the 3D shapes in which surface and boundary were curved along the vertical axis) showed selectivity for the 3D boundaries (decorrelation: N=15, solid shape: N=9). Twenty-two percent of the neurons tested (10/46) were selective for both the 3D boundaries and the 3D surfaces. The remaining 6 neurons required the presence of both surface and boundary in depth. Thus, curved boundaries can be sufficient for 3D shape-selectivity in TEs, but a large proportion of the neurons displays selectivity for 3D surfaces lacking boundary disparities, implying coding of both surface- and boundary information in TEs.

2. Fundamentals of shape representation: the representation of perceptual similarity of shapes in macaque inferior temporal cortex.

A large amount of behavioral research has shown that the perceptual similarity between object shapes is a major determinant of classification and discrimination performance. Behavioral studies also suggest that the human visual system represents metric physical similarities in a faithful way. However, it is still a open question how shapes are represented by macaque inferior temporal neurons and whether this neural representation reflect perceptual and/or physical similarities among shapes.

This question was addressed by measuring the representation of similarities of parametrically varied two-dimensional shapes (Fourier Descriptors) in the anterior IT cortex of two awake rhesus monkeys. The shapes were divided into three 'categories' with distinct radial frequency components, resulting in qualitative shape differences. Within each category, metric differences between shapes were induced by varying independently the amplitude of two radial frequency components, in effect creating 8 shapes for each category arranged in a square-like manner in the two-dimensional amplitude space.

So far, we have recorded from 75 neurons while the monkeys were fixating and discriminating the stimuli. Using the neural responses of these neurons to each of the 24 shapes, a similarity matrix was constructed by calculating the similarity between each stimulus combination in the multi-dimensional space spanned by these 75 neurons. The application of cluster analysis and multidimensional scaling to these similarity data showed that the representation of the shape similarities in the inferior temporal cortex is highly veridical: both the clustering into three categories and the within-category shape arrangement were reflected in the neural similarities. While the responses of many neurons were determined by category membership, there was a surprisingly large proportion of neurons with similar maximum responses for different categories (no clear between-category selectivity), but with good within-category selectivity, fitting our previous results (Vogels, Europ. J. Neurosci., 1999) using more complex images (e.g. trees).

3. Position sensitivity of macaque inferior temporal neurons

Recent findings in dorsal visual stream areas (e.g. parietal cortex) and computational work (e.g. by Edelman and by Biederman) raise the question whether neurons at the end-station of the ventral visual stream can code for stimulus position. We have now provided the first detailed quantitative data on the spatial sensitivity of neurons in the anterior part of the inferior temporal cortex (area TE) in awake, fixating, monkeys. We observed a large variation in receptive field (RF) size (ranging from 2.8º to 26º). TE neurons differed in their optimal position with a bias towards the foveal position. Moreover, the RF profiles of most TE neurons could be fitted well with a two-dimensional Gaussian function. Most neurons had only one region of high sensitivity and showed a smooth decline in sensitivity towards more distal positions.

In addition, we investigated some of the possible determinants of such spatial sensitivity. First, testing with low-pass filtered versions of the stimuli revealed that the general preference for the foveal position and the size of the RFs was not due simply to TE neurons receiving input with a lower spatial resolution at more eccentric positions. The foveal position was still preferred after intense low-pass filtering. Second, although an increase in stimulus size consistently broadened spatial sensitivity profiles, it did not change the qualitative features of these profiles. Moreover, size selectivity of TE neurons was generally position invariant. Overall, our results suggest that TE neurons can code for the position of stimuli in the central region of the visual field. Furthermore, their spatial sensitivity is much larger as was suspected. These results have been published in the *Journal of Comparative Neurology*.

4. Effect of shape discrimination learning on shape representations in inferior temporal cortex: changes in the selectivity of macaque inferior temporal neurons during fast shape discrimination learning.

The shape selectivity of inferior temporal neurons is thought to underlie shape discriminations. One important question is whether this shape selectivity is malleable and whether an improvement in (behavioral) shape discrimination or learning novel shapes changes the shape selectivity. So far, studies reporting changes in the response of IT neurons employed extensive practice programs that lasted at least several weeks. However, it is unclear whether these changes in IT shape selectivity correlate with behavioral improvements. Furthermore, primates show shape discrimination learning in a single session, and, it is not known whether IT cells change their shape selectivity within a single training session, and, if so, whether the latter is correlated with the behavioral learning.

We have determined whether the shape selectivity of macaque TE neurons improves while the animal learns to discriminate minute differences between shapes in a single session. We measured the responses of the neurons while the monkey was learning to discriminate the shapes. Prior to recordings, 2 rhesus monkeys were trained in the discrimination of 10 shapes in a two-choice discrimination task. Responsive neurons were searched while the animal was discriminating these shapes. Once a responsive neuron was found, the animal had to learn to discriminate the familiar shape the neuron responded to from a novel shape that differed slightly from the familiar one. The correct response to the novel shape was the alternative of the one required for the familiar shape (e.g. leftward response for familiar shape .-> rightward for

novel). The response strength of many neurons changed during learning, with 31% of 49 shape selective neurons showing a significant change in shape selectivity. Only 2 neurons showed an increase in shape selectivity with learning. At the population level, the mean absolute difference between the responses to novel and familiar shapes, averaged over all neurons, was not affected by the training, although the behavioral performance measured in the same trials increased from 62% (first 40 trials) to 83% percent correct (after 200 trials). It is highly unlikely that these changes in shape selectivity, observed in a minority of neurons, underlie the improvement in behavioral discrimination since the neuronal changes were also observed in a 3th monkey that was not trained in discrimination but was merely performing a fixation task. These results suggest that although single TE neurons may show changes in shape selectivity when stimulated repeatedly with the same shapes in a single session, these changes are unrelated to behavioral discrimination improvements, but reflect automatic adaptations of shape selectivity.

5. Brain regions involved in categorization of dot pattern in men.

Categorization of dot-patterns (Posner and Keele, J. Exp. Psychol., *77*, 353, 1968) has been widely used to study mechanisms of natural categorization. This paradigm to study categorization employs novel, nonsense dot patterns that are distorted versions of one of several underlying prototypic dot patterns. In each trial, a novel distorted dot pattern is presented and the subject is required to classify it as belonging to one of the categories defined by the prototypes, followed by feedback. However, the subject is not exposed to the prototypes so she/he has to abstract the categories from the individual presentations of the distorted patterns. The advantage of this paradigm is that, on the one hand, the categories are defined probabilistically, like naturally occurring categories (e.g. trees, cars, birds etc.), and, on the other hand, the distance between exemplars of one or different categories is controlled by a known, statistical rule, unlike for exemplars of natural categories.

To determine the brain regions involved in this sort of categorization, we compared brain activation, using PET, in 2 tasks employing 9-dot patterns. In the categorization task, subjects (n = 14) categorized distorted, novel exemplars of 2 categories and random patterns (3 response choices). The control task consisted of a discrimination of pattern position. Each task was run at 2, matched, levels of difficulty, manipulated by the distortion level or position shift size. Yes/no feedback was provided. Fixation of the fixation target served as baseline condition.

The categorization task differentially activated ($z>4.30$; analyzed by SPM96) the lateral orbitofrontal cortex (BA 11) and 2 dorsolateral prefrontal regions (BA 46 and 9). These regions were not activated in the position discrimination task (vs. baseline condition). Also, the categorization task activated a parietal focus (dorsal intraparietal sulcus) and the cerebellum. The position discrimination task activated also the latter regions, but less so than during categorization. Subtracting categorization from position discrimination yielded a middle temporal (BA 20/21) and medial frontal gyrus (BA10) focus. Both regions showed deactivations in the categorization task (vs. baseline). Task difficulty had no effect.

These human imaging results show that the dot-pattern categorization task engages strongly prefrontal areas, which agrees with the results of very recent single cell recordings in the macaque prefrontal cortex showing category-related responses (Freeman et al., Science, in press).

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