Geneeskundige Stichting Koningin Elisabeth

Fondation Médicale Reine Elisabeth

verslag rapport

2005

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Geneeskundige Stichting Koningin Elisabeth

2005

Inleiding Verslag Activiteiten van de GSKE – FMRE

Na een moeilijke selectie van 86 projecten, heeft het wetenschappelijk comité er 17 weerhouden. Deze projecten krijgen gedurende 3 jaren kredieten van de Stichting

Het gaat respectievelijk over de ploegen van de professoren Belachew S. (Ulg)., De Schutter E.(U.A.), Goffinet A. (U.C.L.), Janssen P .(K.U.Leuven), Maquet P .(Ulg), Michotte Y. (V.U.B.), Octave J.N.(U.C.L.), Olivier E.(U.C.L.), Parmentier M. (U.L.B.), Ris L. (U.M.H.), Schiffmann S.N. (U.L.B.), Tavernier J. (Ugent), Timmerman V. (U.A.), Vanderhaeghen P. (U.L.B.), Vanduffel W.(K.U.Leuven), Vanhoenacker P (UGent). en Vogels R..(K.U.Leuven).
Zoals bij iedere selectie, verlengen sommige ploegen hun kredieten, andere worden niet weerhouden en tenslotte worden er nieuwe ploegen geselecteerd. Dit weerspiegelt goed de dynamiek van het onderzoek in het domein van de neurowetenschappen in ons land en de strengheid waarmee het wetenschappelijk comité de dossiers evalueert.

Dit jaar is eveneens een speciaal jaar, want dank zij de vrijgevigheid van mecenassen, Baron van Gysel de Meise, enerzijds en de vennootschap UCB anderzijds, kan het comité een supplementair onderzoekskrediet van respectievelijk 12.500 euro en 25.000 euro toekennen aan onderzoeksploegen die geselecteerd worden op basis van hun activiteiten rapport 2005.
Deze hernieuwbare kredieten dragen de naam van enerzijds " Prijs van Gysel de Meise voor de neuro-wetenschappen " en anderzijds " UCB Award ". De prijzen zullen uitgereikt worden in de lente van 2006.

De Stichting heeft eveneens zijn communicatie middelen ontwikkeld en heeft, dank zij de medewerking van jonge enthousiaste informatici, een internet site gecreëerd in vier talen.
Deze site geeft alle nodige informatie over de historiek, de organisatie en de wetenschappelijke activiteiten van de gesteunde ploegen via hun rapporten van de laatste jaren. De website zal volledig de papieren documenten vervangen.

Deze korte inleiding bij het activiteitenrapport 2005, is ook een gelegenheid om de raad ven bestuur te danken voor hun doorzicht en hun milde steun aan het onderzoek in dit particulier domein als dit van de neurowetenschappen.

Al onze waardering en erkentelijkheid gaat ook naar H.K.H. Prinses Astrid voor haar niet aflatende aandacht en steun door Haar aanwezigheid op alle evenementen die georganiseerd worden door de Stichting. In naam van alle onderzoekers en van het wetenschappelijk comité danken wij Haar hiervoor zeer hartelijk.

Prof. Dr. Th. de Barsy
Wetenschappelijk Directeur
Brussel, maart 2006

Fondation Médicale Reine Elisabeth

2005

Introduction Rapport d'Activités de la FMRE - GSKE

Après une sélection difficile de 86 projets, le comité scientifique en a retenu 17 qui bénéficieront de crédits de la Fondation pendant une période de trois ans.

Il s'agit respectivement des équipes des professeur Belachew S. (ULg), De Schutter E. (U.A), Goffinet A. (U.C.L.), Janssen P. (K.U.Leuven), Maquet P. (ULg), Michotte Y. (V.U.B.), Octave J.N. (U.C.L.), Olivier E. (U.C.L.), Parmentier M. (U.L.B.), Ris L. (U.M.H.), Schiffmann S.N. (U.L.B.), Tavernier J. (Ugent), Timmerman V. (U.A.), Vanderhaeghen P. (U.L.B.), Vanduffel W. (K.U.Leuven), Vanhoenacker P. (Ugent) en Vogels R. (K.U.Leuven).
Comme à chaque sélection, certaines équipes prolongent leurs crédits, d'autres se voient écartées et enfin de nouvelles sont sélectionnées. Ceci traduit bien le dynamisme de la recherche dans le domaine des neurosciences dans notre pays et la rigueur avec laquelle le comité scientifique procède à l'évaluation des dossiers

Cette année est aussi une année particulière, car, grâce à la générosité de mécènes, le Baron van Gysel de Meise d'une part et de la société UCB d'autre part, le comité va pouvoir attribuer un crédit supplémentaire à la recherche d'un montant de 12.500 euros et 25.000 euros respectivement pour les équipes sélectionnées sur base des rapports d'activité de l'année 2005. Ces montants renouvelables portent le nom de " Prix Baron van Gysel de Meise pour les neurosciences " pour l'un et " UCB award " pour l'autre et seront distribués dans le printemps de l'année 2006.

La Fondation a également développé ses moyens de communications et a pu, grâce à l'aide de jeunes informaticiens enthousiastes, constituer un site Internet rédigé en quatre langues. Ce support donne toutes les informations nécessaires sur l'histoire, l'organisation, et les activités scientifiques des équipes soutenues avec les rapports des dernières années.
Ce moyen remplacera entièrement les documents papiers.

Cette brève introduction aux rapports d'activités de l'année 2005 est l'occasion de remercier le conseil d'administration de sa clairvoyance et de sa générosité pour le soutien à la recherche dans un domaine bien particulier qu' est celui des neurosciences.

Toute notre reconnaissance et notre attachement s'adressent à S.A.R. La Princesse Astrid qui ne cesse de porter attention et de soutenir par sa présence tous les moments de la vie de la Fondation. Nous lui exprimons ici au nom de tous les chercheurs et au nom du comité scientifique nos très chaleureux remerciements.

Prof. Dr. Th. de Barsy
Directeur Scientifique
Bruxelles, mars 2006

Universitaire ploegen gesteund door de Geneeskundige Stichting Koningin Elisabeth
Equipes universitaires subventionnées par la Fondation Médicale Reine Elisabeth
University Research groups supported by the Queen Elisabeth Medical Foundation

K.U.Leuven

Prof. Dr. P. Janssen
The functional interaction between dorsal and ventral visual stream areas during 3D object discrimination and grasping.

Prof. Dr. W. Vanduffel
Interactions between areas investigated using awake monkey fMRI.

Prof. Dr. R. Vogels
Coding of action categories in primate cortex.

U.A.

Prof. Dr. E. De Schutter
Experimental analysis of cerebellar coding.

Prof. Dr. V. Timmerman
Molecular genetics and biology of Charcot-Marie-Tooth neuropathies.

U.C.L.

Prof. Dr. A. Goffinet
Genetic, molecular and cellular mechanisms of cortical development.

Prof. Dr. J.N. Octave
Phosphorylation of the amyloid precursor protein intracellular domain: regulation of the production of ß-amyloid peptide and transcriptional activity?

Prof. Dr. E. Olivier
Distinct contribution of parietal and frontal cortical areas to the control of finger and hand movements.

U.Gent

Prof. Dr. J. Tavernier

Evaluation of leptin antagonists for treatment of multiple sclerosis.

Prof. Dr. P. Vanhoenacker

The human 5-HT$_7$ receptor: a new link and potential therapeutic target for Alzheimer's disease?

U.L.B.

Prof. Dr. M. Parmentier

Characterization of the role of G protein-coupled receptors in the central nervous system by using genetically invalidated mouse models.

Prof. Dr. S.N. Schiffmann

Roles of the direct and indirect pathways in functions and disorders of the basal ganglia.

Dr. P. Vanderhaeghen

Developmental mechanisms patterning neuronal connectivity in the cerebral cortex.

U.Lg

Dr. S. Belachew

Unraveling the role of type 2 cyclin-dependent kinase during inner ear and white matter development.

Prof. Dr. P. Maquet

Human Brain Function in sleep. Studies in man by multimodal functional neuroimaging.

U.M.H.

Dr. L. Ris

Role and Mechanisms of synaptic "Tagging" in long-term memory.

V.U.B.

Prof. Dr. Y. Michotte

In vitro and in vivo studies on the role of the IRAP enzyme/AT$_4$ receptor system in learning and memory processes.

Progress Reports of the University Research Groups, supported by the Queen Elisabeth Medical Fondation in collaboration with the following Professors and Doctors (2005)

Progress Report of the Research Group of

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Unraveling the role of type 2 cyclin-dependent kinase during inner ear and white matter development.

A. Aims

Recent studies have provided evidence that the development of the oligodendroglial lineage in the central nervous system (CNS) and the maturation of hair cells (HCs) in the mammalian organ of Corti do share common regulatory mechanisms through the complex interactions between cyclins and cyclin-dependant kinases (cdks) (Belachew et al., 2002;Malgrange et al., 2003;Ghiani and Gallo, 2001). Both in HCs and oligodendrocytes, the fine tuning of cell cycle exit and the onset of differentiation appear to be linked to cyclin/cdks-dependent molecular pathways in vitro. In the present project proposal, we will try to unravel the functional role of type 2 cyclin-dependent kinase (cdk2) in HCs and oligodendroglial maturation in vivo using a transgenic mouse line which is deficient for cdk2. Improving our understanding of the role of cdk2 with respect to the development of the inner ear sensory epithelium and CNS white matter will prove to be crucial for designing new strategies of treatment for hearing and myelin disorders.

- Oligodendrocytes are the myelin-forming cells of the CNS. They derive from progenitor cells that undergo a series of complex developmental stages to attain a mature myelinating phenotype (Pfeiffer et al., 1993). The progression from progenitors to myelinating oligodendrocytes entails a sequence of events, including cell cycle withdrawal, onset and progression of a differentiation program in which cytoskeletal changes and synthesis of myelin components occur. The end-point of differentiation is the formation of myelin sheaths around multiple axons that facilitates saltatory nerve conduction (Franklin, 2002c). Although the majority of oligodendrocyte progenitor cells (OPCs) develop into oligodendrocytes, some persist in the adult CNS as a population of slowly dividing precursor cells (Ffrench-Constant and Raff, 1986;Wolswijk and Noble, 1989b;Reynolds and Hardy, 1997) that are thought to be the major cellular reservoir responsible for remyelination after white matter insults (Gensert and Goldman, 1997;Blakemore and Keirstead, 1999;Chang et al., 2000;Chang et al., 2002;Franklin, 2002b). Several lines of evidence indicate that adult OPCs, though seemingly recalcitrant, retain the innate capacity to proliferate and differentiate into mature oligodendrocytes when coaxed by appropriate culture conditions (Shi et al., 1998;Wolswijk and Noble, 1989a). In experimental animal models, remyelination indeed occurs with high efficiency (Linington et al., 1992;Nait-Oumesmar et al., 1999). Unfortunately, this is not the case in several human demyelinating diseases, including multiple sclerosis and leukodystrophies. The causative factors in the failure of remyelination are unknown, though probable explanations lie in defective OPC proliferation or survival (Franklin, 2002a;Sim et al., 2002).

- One in 10 individuals is affected by hearing disorders. Hearing impairments can be caused by a variety of factors including noise, ototoxic substances such as aminoglycosides, and aging. In the mammalian organ of Corti, these factors affect the hair cells (HCs) that transduce auditory signals to the brain via the spiral ganglion neurons. Hearing deficiencies are caused by the loss of HCs or spiral ganglion neurons and these changes used to be irreversible. HCs are not

capable of post-embryonic proliferation to produce new HCs. It is therefore necessary to develop strategies aimed at preventing or repairing damaged HCs. Mature sensory organs of the vertebrate inner ear contain two major cell types, mechanosensory hair cells and supporting cells, which share a common origin. Permanent hearing and balance deficits usually occur in humans via the loss of hair cells. Approximately 10% of the population suffers from hair cell loss due to acoustic trauma, treatments with antibiotics, infections or aging. In some vertebrates such as fish and birds, loss of damaged hair cells leads to regeneration of these cells from the supporting cells, and to a recovery of the auditory or balance function. In the mammalian inner ear, hair cell loss has been considered irreversible with no spontaneous hair cell regeneration. The low potential of de-differentiation and proliferation of mammalian supporting cells is thought to limit regeneration in mammalian inner ear.

Very little is known about the molecular pathways that control the proliferation and differentiation of OPCs and inner ear sensory hair cell precursors. We plan to build up here a collaborative task-force combining specific skills of two groups headed by Brigitte Malgrange and Shibeshih Belachew, in order to develop a multidisciplinary cellular and molecular approach to investigate the two following aims, which are apparently distinct but actually converge onto similar developmental issues that we address in two different tissue systems:

In the first year of this project, we have addressed the first of our two aims:

Aim 1: To elucidate the role of cdk2 in vivo during normal white matter development and in a model of acquired demyelination.

Aim 2: To dissect out the functional involvement of cdk2 during inner ear hair cell differentiation

B. Results and Perspectives:

Type 2 cyclin-dependent kinase (Cdk2), which controls G1/S transition in eukaryotic cell cycle, was recently shown to be dispensable during embryonic development, since cdk2-null mice develop normally until adulthood. We assessed here the requirement of Cdk2 for proliferation of CNS precursor cells that generate newborn oligodendrocytes, but also neurons in specific regions of the adult brain. We analyzed subcortical white matter, corpus callosum, striatum and cerebellar white matter areas with a broad spectrum of antigenic markers for distinct stages of oligodendroglial maturation (CNPase, MAG, Olig2). We found that the density and distribution of CNPAase-, MAG- and Olig2-expressing cells was identical in the corpus callosum and sub-cortical white matter of wild-type (WT) versus $Cdk2^{-/-}$ adult mice (3-months old) (Figure 1). Similar results were obtained from the cerebellar white matter and striatum (Figure 2). Finally, the absolute density of Olig2-expressing cells (i.e. the entire oligodendroglial lineage cells) in the corpus callosum was not different between WT and $Cdk2^{-/-}$ adult mice (Figure 3). However, the density of NG2-expressing oligodendrocyte precursor cells (OPCs) was decreased in the corpus callosum (Figure 4) and subventricular zone (SVZ) (data not shown) of

adult Cdk2-null mice. Altogether, these data provide evidence that Cdk2 does not appear to be essential for normal developmental myelination, but the pool of adult OPCs is depleted in adult $Cdk2^{-/-}$ mice. This indicates that the function of Cdk2 may be effectively compensated in neonatal OPCs whereas it could be crucial for cell cycle kinetics in adult OPCs. Hence, we will assess the functional consequences of Cdk2 deletion on OP cell proliferation at different postnatal stages. We will also appraise the role of Cdk2 in the rate of oligodendrogenesis in the uninjured white matter. Finally, we will study to what extent Cdk2 deficiency could impede myelin repair after lysolecithin-induced demyelination or in a model of experimental autoimmune encephalomyelitis (EAE).

Besides the role that Cdk2 plays in the control of OP cell proliferation, we have analyzed proliferative events in the adult neurogenic areas, since $p27^{kip1}$, which is the principal endogenous blocker of Cdk2, inhibits adult neurogenesis (Doetsch et al., 2002). We assessed the anterior subventricular zone (aSVZ), the lateral SVZ (LV) and rostral migratory stream (RMS) in the P90 $Cdk2^{-/-}$mouse. Immunolabeling with anti-BrdU and anti-Ki67 antibodies after 2 BrdU injections revealed that, in the $Cdk2^{-/-}$ mouse, the percentage of $BrdU^+$ and $Ki67^+$ cells in the aSVZ, LV and RMS was lower than in the $Cdk2^{+/+}$ mouse (Figure 5). $NG2^+$ cell proliferation was also significantly reduced in the aSVZ, LV and RMS

areas of adult $Cdk2^{-/-}$ mice (Figure 6). To further characterize $NG2^+$ cell development, we used a broad spectrum of antigenic markers: Lex, nestin, PSA-NCAM, Nkx2.2 and GFAP. We have recently demonstrated that, in $Cdk2^{+/+}$ mice, aSVZ progenitors are multipotent, and can generate oligodendrocytes and interneurons in the postnatal olfactory bulb (Aguirre and Gallo, 2004). In $Cdk2^{-/-}$ mice, we observed a reduction in the number of undifferentiated progenitors ($NG2^+$/$nestin^+$) in the aSVZ, LV and RMS, as compared to WT (Figure 7). In the same brain regions of the $Cdk2^{-/-}$ mouse, a larger percentage of $NG2^+$ cells expressed PSA-NCAM, as compared to $Cdk2^{+/+}$ animals, suggesting that more $NG2^+$ cells became postmitotic neuroblasts (Figure 8). None of the $NG2^+$ cells was $GFAP^+$, and only a small population of $NG2^+$ cells co-expressed Nkx2.2. These results, suggest that Cdk2 not only regulates proliferation in the SVZ-RMS-olfactory bulb pathway but could also have non cell cycle-related functions in fate determination and lineage progression in this area. Our future work will characterize the full range of effects of Cdk2 deletion with respect to neurogenesis and fate specification in this region.

Besides the SVZ-RMS, we have found that the density of S-phase dividing progenitors was significantly decreased in the dentate gyrus of adult Cdk2-null mice, where neurogenesis also persists throughout life. Consistently, the total amount of adult-born granule neurons in the dentate gyrus was drastically reduced in Cdk2-null mice (Figure 9).

Altogether, our data showed that Cdk2: i) is not required for normal postnatal myelination of the CNS, ii) controls adult OP cell density in white matter areas, and thus could impede myelin repair after demyelination, iii) regulates proliferation of neural progenitor cells in the SVZ/RMS germinative areas, and iv) controls neural progenitor cell proliferation and neurogenesis in the adult hippocampus.

C. Figures :

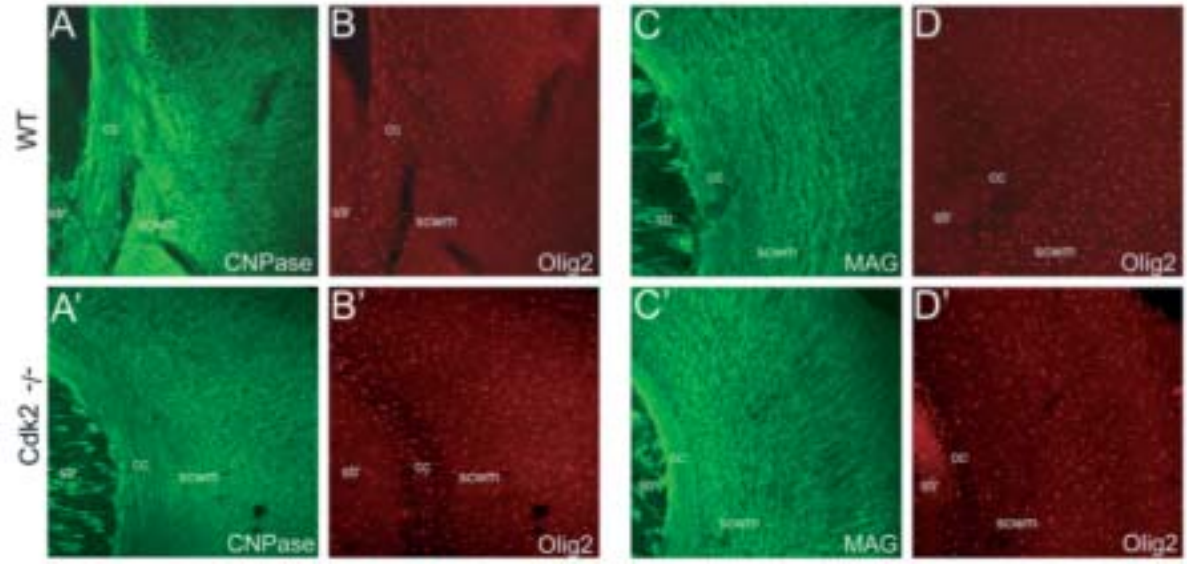

Figure 1: (A-D and A'-D') Single plane confocal images of subcortical whitte matter (scwm), striatum (str), and corpus callosum (cc) areas from P90 wild-type (WT) (A-D) and Cdk2-deficient mice (Cdk2-/-) (A'-D'). Images were obtained from 50 µm-thick coronal sections immunostained for CNPase, MAG and Olig2. A-B, A'-B', C-D and C'-D' represent the same microscopic fields.

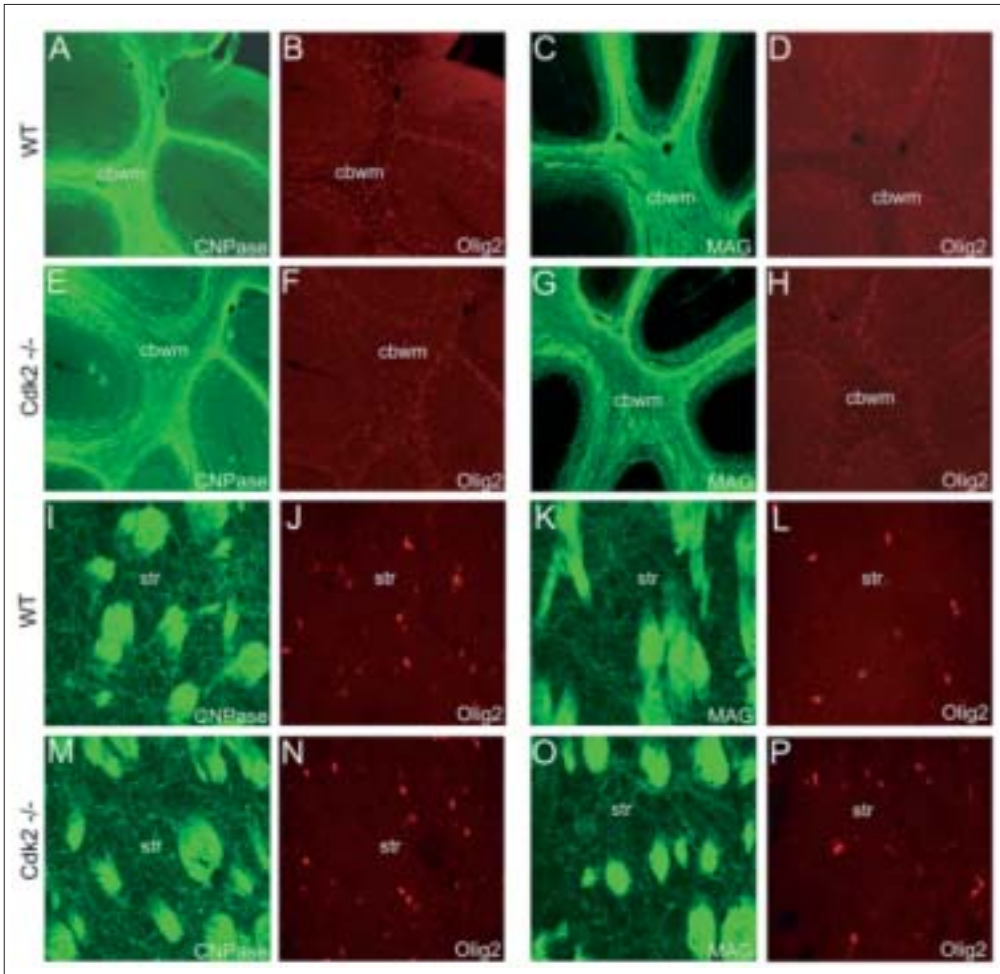

Figure 2: (A-P) Single plane confocal images of cerebellar white matter (cbwm, A-H) and striatum (str, I-P) areas from P90 wild-type (WT) (A-D, I-L) and Cdk2-deficient mice (Cdk2-/-) (E-H,M-P). Images were obtained from 50 µm-thick coronal sections immunostained for CNPase, MAG and Olig2. A-B, C-D, E-F, G-H, I-J, K-L, M-N, O-P represent the same microscopic fields.

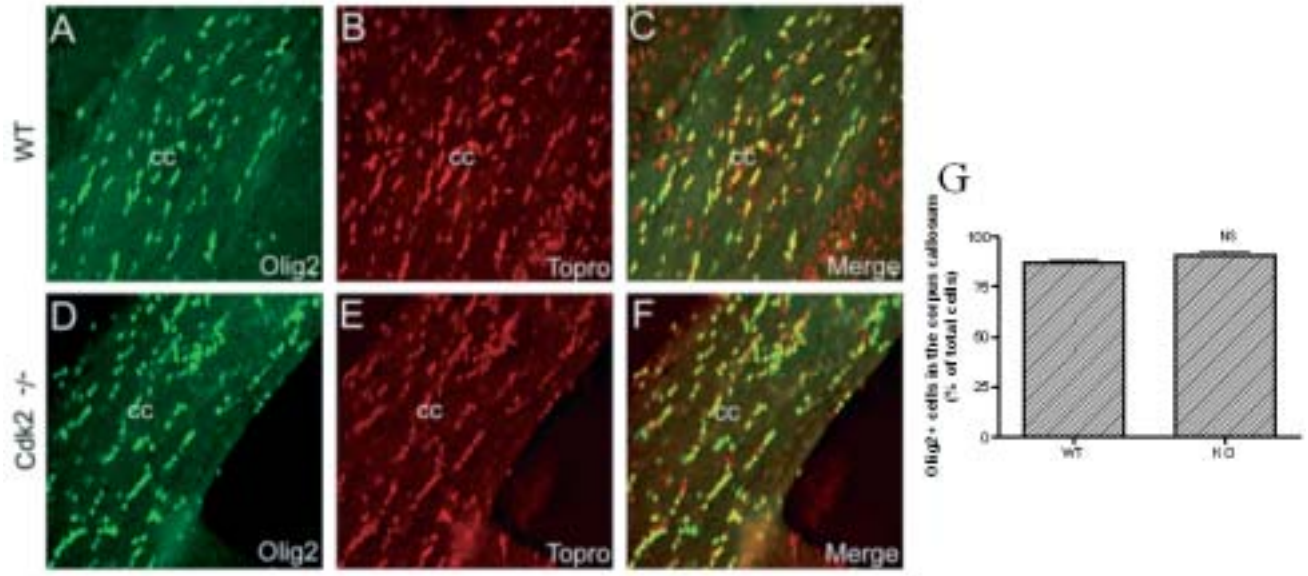

Figure 3: (A-F) Single plane confocal images of the corpus callosum (cc) area from P90 wild-type (WT) (A-C) and Cdk2-deficient mice (Cdk2 -/-) (D-F). Images were obtained from 50 µm-thick coronal sections immunostained for Olig2. Topro-3 was used for nuclear counterstaining. (G) We assessed the overall density of total oligodendroglial lineage cells in the corpus callosum, i.e. Olig2+ cells (% of total cells) (mean ± SD, ANOVA1, Dunnet's post-test, NS= not significant).

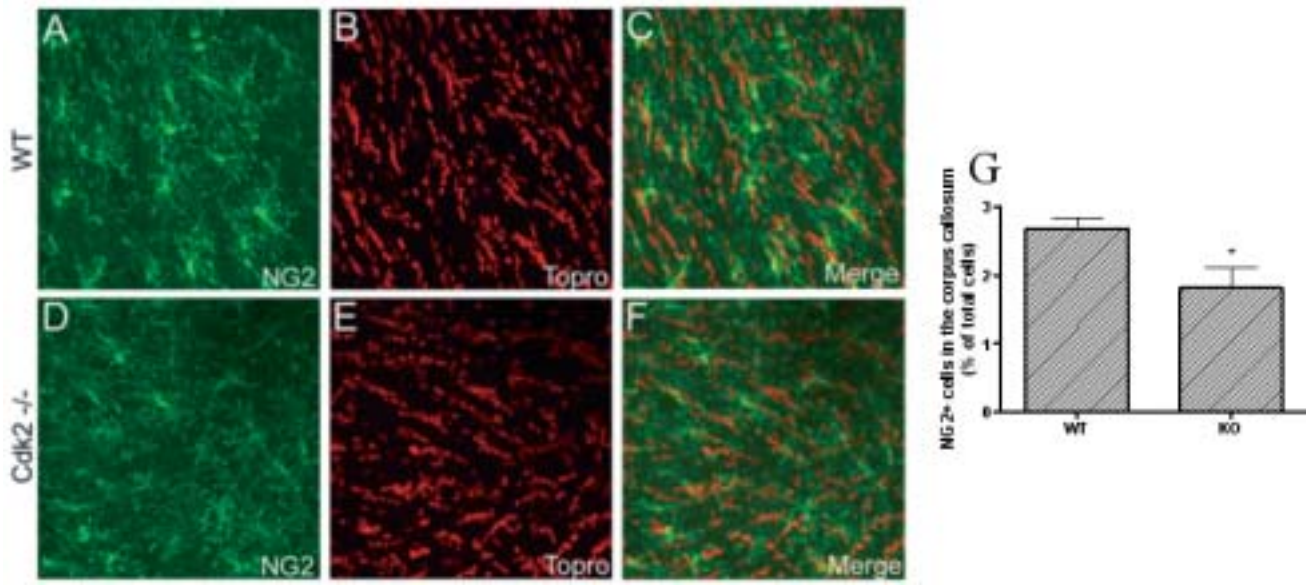

Figure 4: (A-F) " Z-Stack " projection of confocal images in the corpus callosum area from P90 wild-type (WT) (A-C) and Cdk2-deficient mice (Cdk2-/-) (D-F). Images were obtained from 50 µm-thick coronal sections immunostained for NG2 ((white arrows points towards NG2+ cells).Topro-3 was used for nuclear counterstaining. (G) We assessed the overall density of OPCs in the corpus callosum, i.e. NG2+ cells (% of total cells) (mean ± SD, ANOVA1, Dunnet's post-test, *p<0,001).

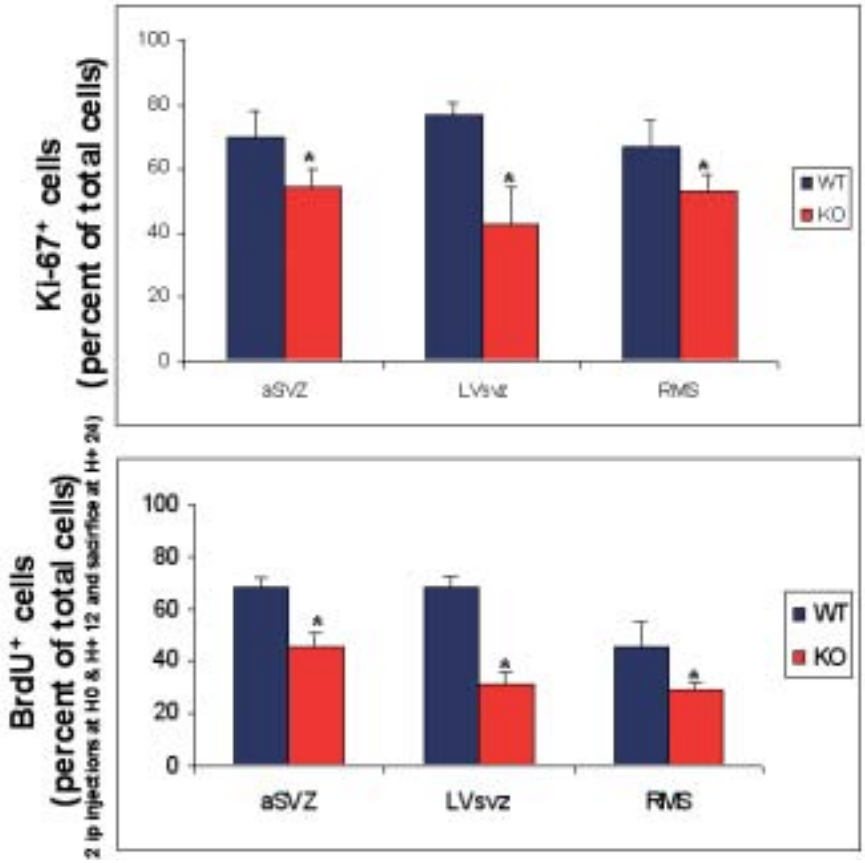

Figure 5: Total neural progenitor cell proliferation in WT versus Cdk2-/- mice

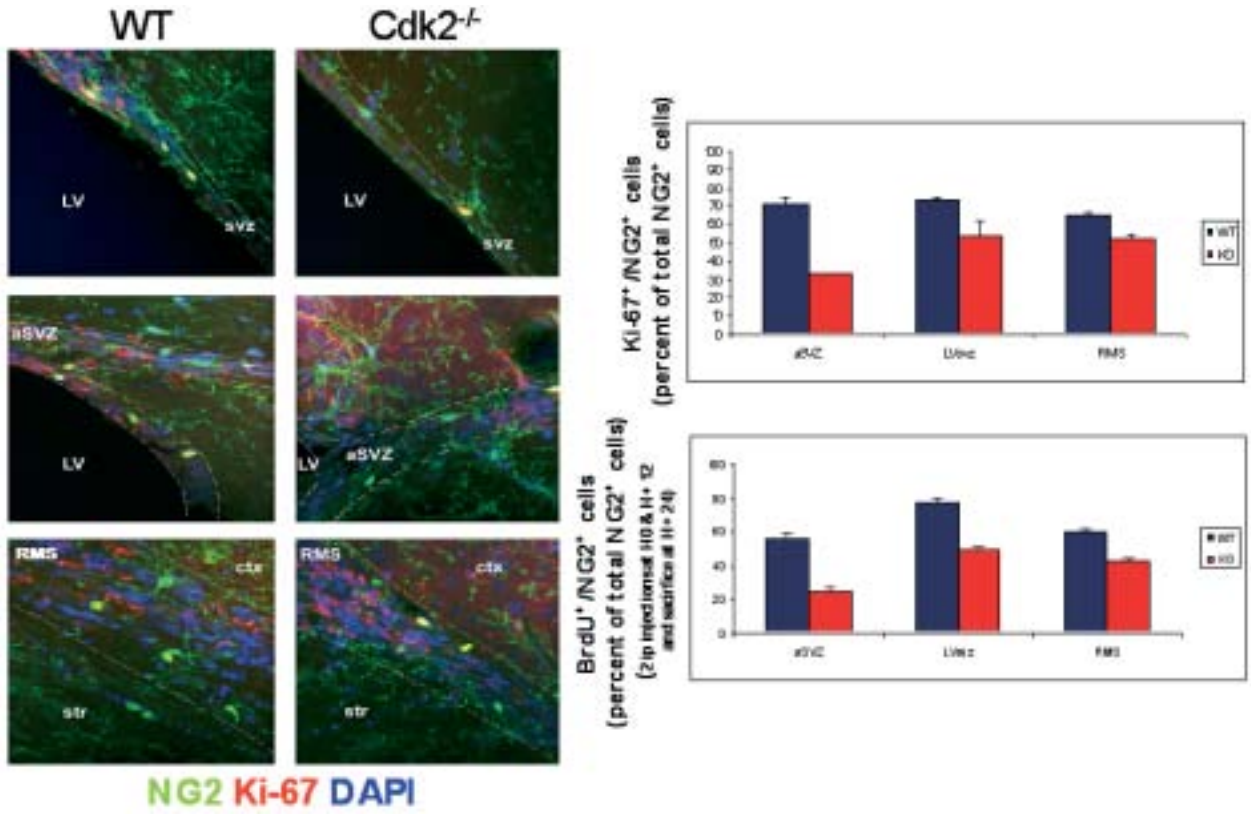

Figure 6: Decreased proliferation in NG2+ progenitor cells from adult Cdk2-/- mice.

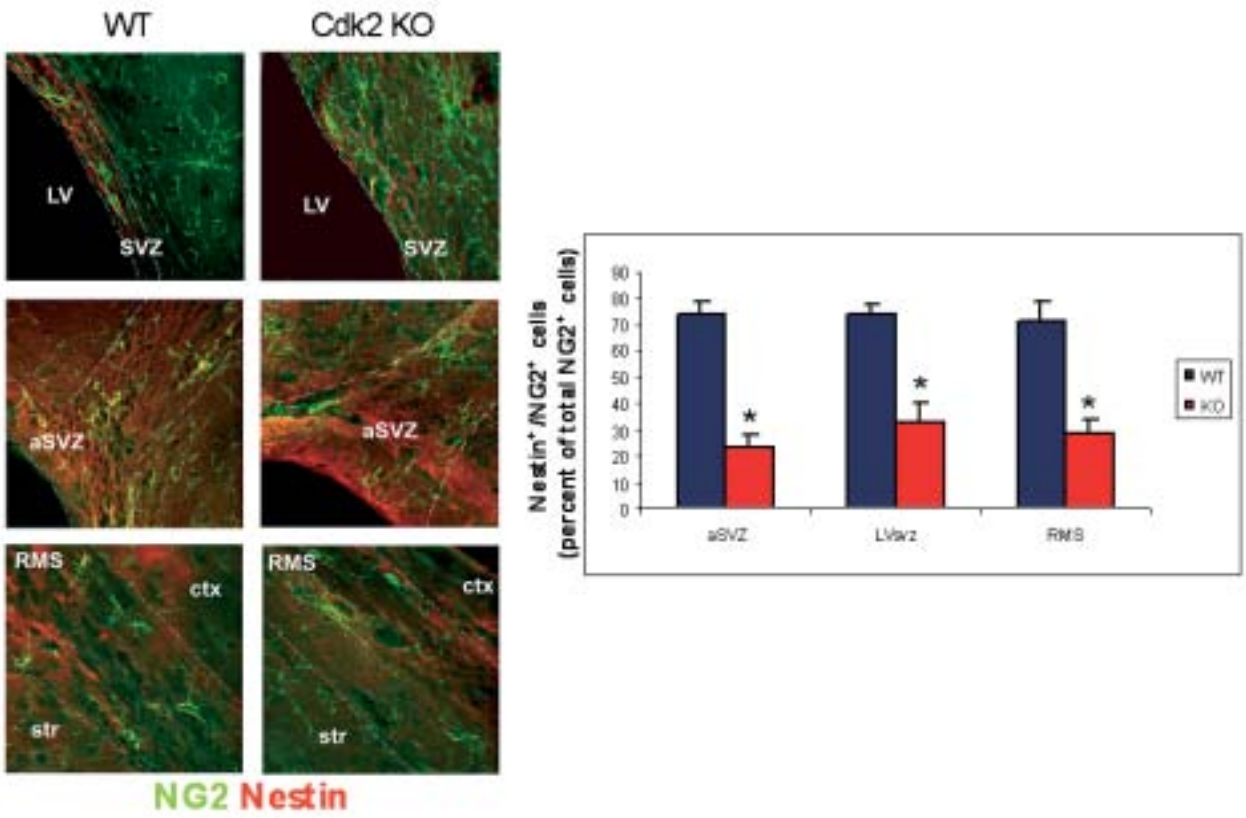

Figure 7: Cdk2 deletion decreases the proportion of NG2$^+$ progenitor cells expressing the neural stem cell marker nestin.

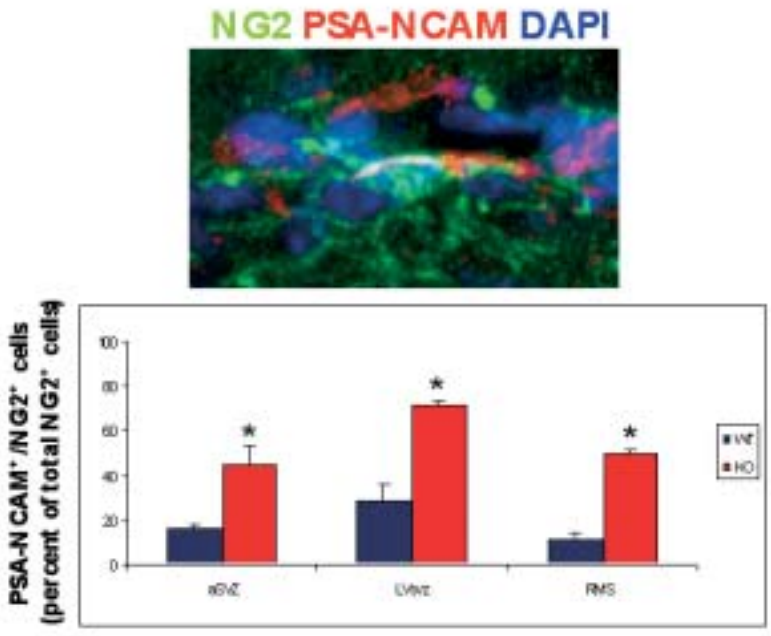

Figure 8: Cdk2 deletion increases the proportion of NG2$^+$ progenitor cells expressing PSA-NCAM.

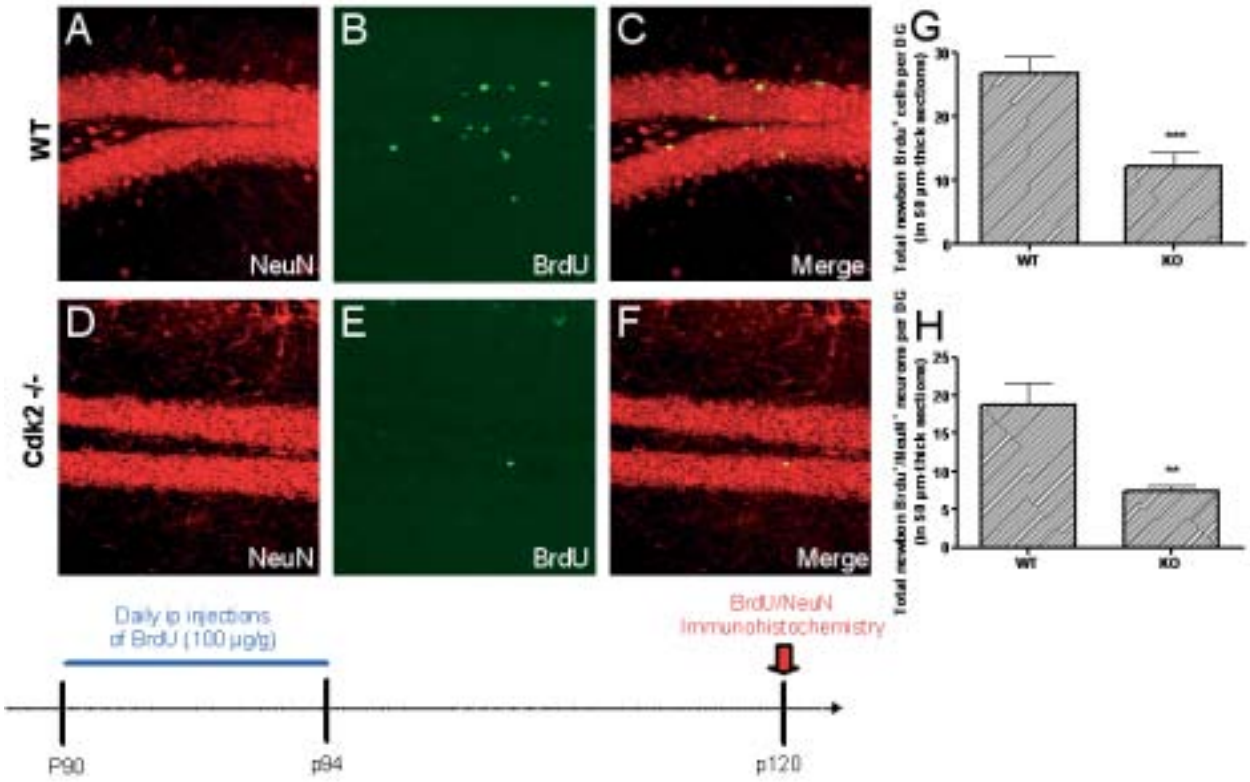

Figure 9: Neurogenesis is decreased in the dentate gyrus of adult Cdk2$^{-/-}$ mice.

D. References

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Progress Report of the Research Group of

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Christoph Tombeur
Ken Veys

Experimental analysis of cerebellar coding.

Dynamic synchronization of Purkinje cell simple spikes

Purkinje cells (PC) generate two types of spikes, simple spikes (SSs) and complex spikes (CS). High frequency SS are driven by parallel fibers (PFs) originating from diverse brain stem areas and pontine nuclei, while complex spikes (CSs) are generated by climbing fibers (CFs) originating from sole inferior olive. Despite the large overlap of PF inputs impinging on PCs lying along the same PF beam, SSs do not show any precise synchronization in PCs separated by more than 500 µm. Conversely, precise synchronization of SS spikes has been reported in pairs of close by PCs, either on the same electrode or two electrodes separated less than 100 m.

To elucidate the mechanism underlying precise synchronization of SSs we have analyzed crosscorrelations of nearby PCs in detail. We report here that only SS pauses are precisely synchronized while other spikes show broad correlations reported. Pauses in SS trains can be generated by multiple mechanisms. It has been reported that CSs are followed by pauses lasting from 15 ms up to several hundred milliseconds. On the other hand, pauses can also be generated by intrinsic afterhyperpolarization, decreased PF input, and/or increased inhibition from surrounding interneurons. All these pauses would be indistinguishable in spike trains recorded from PC axons contacting downstream neurons in the deep cerebellar nucleus (DCN). We define pauses in this study as all ISIs longer than the minimal length of pauses observed after CSs.

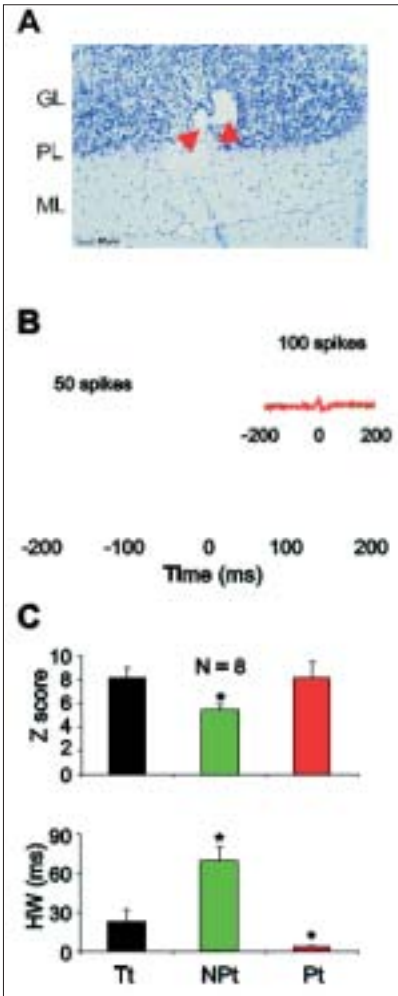

Fig. 1: Synchronized pairs of PCs

We investigated the synchronization of nearby Purkinje cells in ketamine-xyzaline anesthetized rats by inserting two wire electrodes in cerebellar cortex. In 8 pairs of PCs separated by 69.8 ± 9.4 µm (range: 50 - 100 µm, Figure 1A) we found evidence of tight synchronization of all SS (Z score 8.2 ± 0.9, range: 4.6 - 11.5; half-width 22.3 ± 10.1 ms, range: 2 - 84 ms; Figure 1B-C black), which confirms previous. In all cases the peak on the crosscorrelogram consisted of a sharp peak (precise correlation) riding on top of a broader central peak (loose correlation).

We hypothesized that these two peaks reflected the differential synchronization of specific components of the SS train and developed a spike separation method based on the length of the ISI to separate these two components. Specifically we categorized the SS in pause and non-pause ISIs. As it has previously been reported that CSs are often followed by pauses we used a threshold for the length of ISIs that maximized the detection of

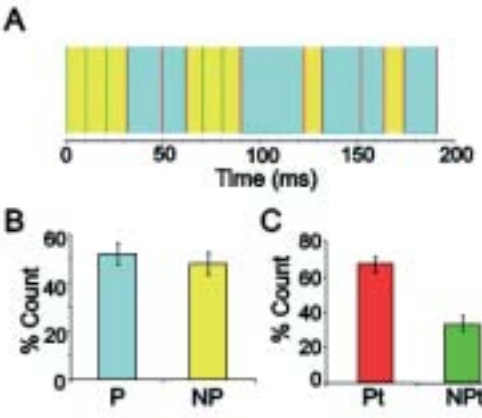

Fig. 2: Pauses and pause timings

pauses following CSs (n =38 PCs). This resulted in the definition of a pause as any interval equal to or larger than 12 ms. In the 8 PC pairs, 52.5 ± 4.5 % of ISIs were pauses (Figure 2B), which was not different from single cell recordings ($p > 0.9$, Student's t test). Using this criterion spikes were classified as either pause time (Pt, spikes beginning, surrounding, or ending a pause) or non-pause time (NPt, the rest of the spikes) (Figure 2A). In the 8 PC pairs 73.2 ± 4.1 % of spikes were classified as Pt (Figure 2C).

Using this criterion we recomputed the cross-correlogram for either all Pt spikes (Figure 1B-C, red) or all NPt spikes in both cells of the pairs (green). We found that the sharp peak of the cross-correlogram consisted exclusively of Pt spikes (Z score 8.1 ± 1.3, range: 3.9 - 15.9; half-width 4.0 ± 0.7 ms, range: 2 - 8 ms), while the NPt spikes caused the broad peak (Z score 5.4 ± 0.5, range: 3.1 - 7.2; half-width 70.0 ± 10.6 ms, range: 40 - 130 ms). These results suggest that only the pauses in the SS trains were precisely synchronized. Overall 17.0 ± 1.4 % of Pt spikes were synchronized (see Methods).

As a control we cross-correlated the Pt of one PC with the NPt of the other PC. These spikes showed no synchrony or a negative correlation, and made no contribution to the short- and long-duration correlation of the overall crosscorrelogram (Figure 1B insets, red and green dotted lines). Next, we investigated whether subgroups of the Pt spike category might be more synchronized than others. Spikes starting (sPt), surrounded by (iPt), or ending (ePt) pauses did not show difference in synchronous pattern compared to each other (Figure 3, $p > 0.4$, Wilcoxon signed rank test). Similarly, there was no preferential length for synchronized pauses above the threshold of 12 ms. As a final control we investigated whether pauses following CSs were responsible for the synchronization observed. We correlated SS spike trains were the first SSs following CSs (pauses following CSs) were removed in either one or both SS trains of pairs where CSs were well discriminated (n = 4, mean CS firing rate = 0.9 ± 0.1). This did not change the peak Z score or half-width of the sharp central peak ($p > 0.6$, Wilcoxon paired rank test, data not shown), which suggests that the precise synchronization shown in Pt was not caused by CSs.

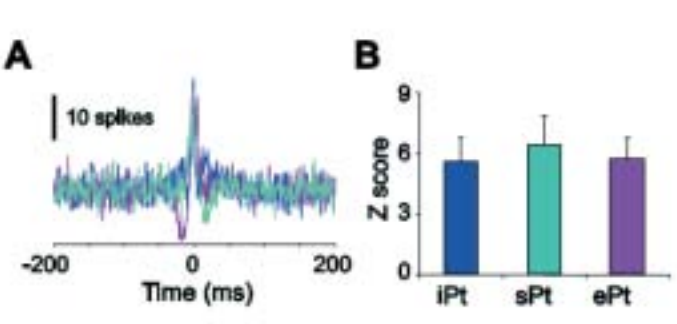

Fig. 3: Cross-correlation of spikes surrounded by starting or ending pauses

Taken together, in this study, we have shown that (1) around half of SS intervals are pauses defined as ISIs of 12 ms or longer, (2) pairs of PCs closer than 100 m

fire 17% of pauses together with a fine temporal precision, (3) faster ISIs also tend to occur together but with much less temporal precision, (4) CSs do not cause the precise synchronization between pauses.

Temporal characteristics of tactile stimuli influence the response profile of cerebellar Golgi cells

As the main inhibitory units of the cerebellar granular layer, Golgi cells play an important role in the preprocessing of afferent information (De Schutter et al., 2000). A distinctive feature of these neurons is the combined trigeminocerebellar and corticopontine inputs they receive, which enable a direct comparison of the information processing in the two pathways (Morissette et al., 1996; Vos et al., 1999; Vos et al., 2000).

The present work focused on the temporal aspect of tactile stimulation, specifically the stimulus duration and frequency of stimulation.

Following the classification introduced in our previous work (Vos et al., 1999), the majority of the Golgi cells (17/31) responded to the punctate stimulus with both an early (8.21 +/- 2.87 ms) and a late (17.37 +/- 3.1 ms) excitatory component, mostly caused by input via the trigeminocerebellar and corticopontine projections, respectively (Morissette et al., 1996; Vos et al., 1999). The response of Golgi cells included a period of decreased activity, or "silent period" which started at 34 +/- 3.5 ms (mean +/- std) and lasted for 200 +/- 78 ms. In a large number of Golgi cells (20/31), the silent period was followed by a period of rebound activity starting at 203 +/- 60 ms after the stimulus onset and ending at 382 +/- 87 ms.

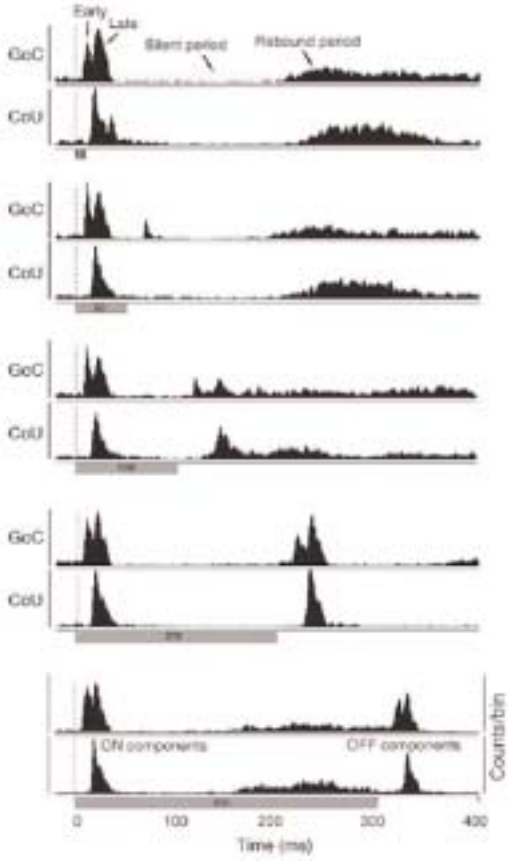

Fig. 4: Example of stimulus-evoked responses in a simultaneously recorded Golgi cell (Goc) and SI cortical unit (CoU)

In response to increasing stimulus durations (first paradigm) an OFF component evoked by the stimulus offset appeared in all of the recorded Golgi cells; this OFF component was not present at 10 ms. In analogy with the early and late ON peaks, the OFF response was composed of early and/or late components. The origin of the late OFF peak was investigated by simultaneously recording SI cerebral cortical units (n = 8, in 5 animals) and cerebellar units with overlapping receptive fields (Fig. 4). The Golgi cell responded to the shortest stimulus (10 ms) with both early and late ON components (Fig. 4, top frame). Increasing the stimulus duration to 50 ms resulted in a small and single OFF component, evoked only in the Golgi cell

(second frame). A further increase to 100 ms evoked a late OFF component in addition to the early one in the Golgi cell, and a single OFF component in the cerebral cortical unit. Thus, the appearance of the late OFF cerebellar component coincided with the occurrence of the cerebral cortical OFF component. Furthermore, we observed that OFF components were only evoked if the corresponding ON responses were also present.

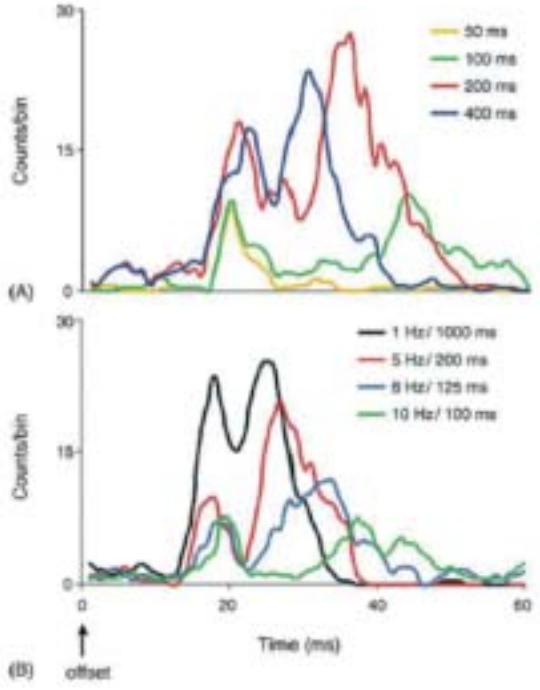

Fig. 5: Similarity between the effect of stimulus duration and interstimulus interval

While the ON responses remained constant for increasing stimulus duration, the OFF responses changed in both amplitude and latency (see infra). We compared these changes to the sensitivity of ON responses of Golgi cells to increasing stimulus frequency (second stimulation paradigm). We observed that the OFF response profile of Golgi cells to an increasing stimulus duration was similar to the ON response profile to a decreasing stimulation frequency (Fig. 5). The ON response profile to a high frequency stimulus (Fig. 5B; 10 Hz) is comparable to the OFF response profile of a short stimulus duration (Fig. 5A, 100ms, ON responses are omitted).

The dependence of the response amplitudes for the cerebellar and cerebral cortical populations on stimulus duration is depicted in Fig. 6. Increasing the stimulus duration did not affect the amplitude of the ON components (fig. 6A). The corresponding OFF responses showed a different profile (Fig. 6B). The responses to the stimulus offset gradually increased for increasing stimulus durations (r^2 > 0.4, P < 0.001, for the 3 curves, stimulus duration 30-200 ms), before reaching a plateau at ~ 200 ms. Interestingly, the initial epoch of ~ 200 ms of small OFF amplitudes mirrored the timing and duration of the silent period evoked by a single short stimulation in Golgi cells. Increasing interstimulus intervals resulted in a similar modification of the early and the late ON response components (Fig. 6D).

The latencies of cerebellar and cerebral cortical ON components were independent of the stimulus duration (r^2 < 0.02, P > 0.1, regression analysis, Fig. 6C), with mean latencies in agreement with previous observations (Vos et al., 1999). The latency of the early OFF component, although significantly longer (mean latency: 16.48 +/- 0.59 ms), showed the same level of stability. In contrast, the latencies of both the late cerebellar and the cerebral cortical OFF components clearly decreased for increasing stimulus duration (r^2 > 0.4; P < 0.0001, stimulus duration 50-250 ms, Fig. 6C) before stabilizing for durations > 300 ms (mean latency: 25.69 +/- 1.09 ms for cerebellar units, 19.98 +/- 0.69 ms for cerebral cortical units, stimulus duration 300-700 ms). Again similar trends were observed when latency values for both early and late components were plotted against the interstimulus intervals (Fig. 6E).

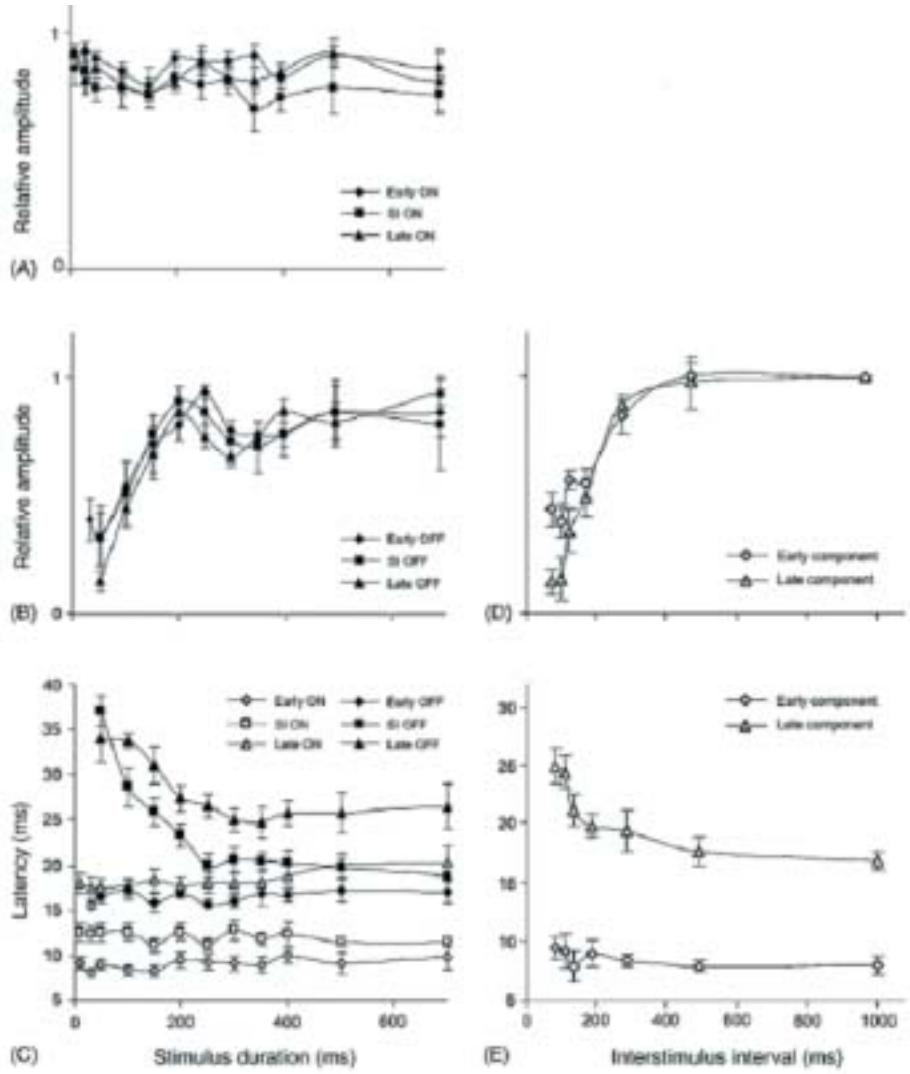

Fig. 6: Effects of temporal characteristics on the response profile of cerebellar Golgi cells and SI cortical units.

Whatever its origin may be, interestingly the silent period seemed to affect the two stimulation protocols we used in a comparable manner. Increasing the stimulus duration or the interstimulus interval resulted in both a latency decrease of the corticopontine responses and an amplitude increase of both responses. However, the response profiles were slightly different for the latencies and the amplitudes, suggesting that amplitude and latency transformations might follow different processes.

In conclusion this study demonstrates that the silent period observed in Golgi cells has a strong effect on both offset responses for short stimulation durations and onset responses to high frequency stimuli. This suggests that the corresponding 200 ms time window may be important in cerebellar processing, at least for somatosensory stimuli.

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Publications 2005 (experimental section):

- K. Tahon, A. Volny-Luraghi, E. De Schutter: Temporal characteristics of tactile stimuli influence the response profile of cerebellar Golgi cells. Neuroscience Letters 390: 156-161 (2005).
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Progress Report of the Research Group of

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Genetic, molecular and cellular mechanisms of cortical development

Background

Work in our laboratory is is focused on: i) A better characterization of the Reelin signaling pathway and of the control of reelin expression; (ii) The use of an *in vitro* system to study neuronal migration to the cortex and the formation of the cortical plate; (iii) Studies of the seven pass cadherin Celsr3, a protein involved in cortical development.

1. The Reelin signaling pathway.

The cloning of the Reelin gene, allowed the definition of a new signaling pathway that plays a key role in organizing the radial and laminar organization of the cortex (Tissir et al., 2002a; Tissir and Goffinet, 2003). Reelin is an extracellular matrix protein secreted by Cajal-Retzius cells (CR), that binds to two receptors of the lipoprotein receptor family named Very Low Density Lipoprotein Receptor (VLDLR) and Apolipoprotein-E Receptor Type 2 (ApoER2) expressed on the surface of migrating neurons. The cytoplasmic adapter Disabled-1 (Dab1) docks to the cytoplasmic tail of receptors and becomes tyrosine phosphorylated by Scr family kinases following Reelin binding to receptors (Jossin et al., 2003). Dab1 activation is further relayed in cortical neurons by various signaling pathways, particularly the PI3K, PKB/Akt (Bock et al., 2003) the Nck-beta and the CrkL-C3G-Rap1 pathways.

Reelin is processed *in vivo* into several products that result from partial cleavage at two sites located respectively between repeats 2 and 3 and between repeats 6 and 7. We showed that the central fragment (repeats 3-6) is necessary and sufficient to fulfill Reelin's function during brain development (Jossin et al., 2004). In order to study better the role of the central part of Reelin in signaling, and after several failures, we could generate new monoclonal antibodies that reacts specifically with that fragment. Two antibodies have been characterized extensively and allowed us to detect the predicted central fragment of reelin in tissues and in human cerebrospinal fluid. We have indication that these antibodies block the reelin signal. As soon as the activity of these antibodies is confirmed, this work will be published

In collaboration with the laboratory of J. Herz (Dallas), we showed that the PI3K and PKB/Akt kinases are implicated in Reelin signaling (Bock et al., 2003). An extensive study of this signaling pathway has been carried out and shows that PI3K mediates both reelin-dependent as well as reelin-independent developmental events. Akt/PKB is regulated by Reelin, like Gsk3beta and mTor, but none of them is involved in mediating the effects of reelin on cortical plate development. These studies of the reelin signal have been submitted for publication (Jossin and Goffinet, 2006).

2. Study of neuronal migration to the cortical plate in vitro

Until recently, no *in vitro* system allowed the analysis of cortical plate (CP) development in vitro from the preplate stage. By systematic optimization of tissue culture parameters, we set up a system in which vibratome slices are prepared at the preplate stage (E13) and cultured for two days *in vitro*. In these conditions, CP development proceeds *in vitro* with features that recapitulate its development *in vivo* (Jossin et al., 2003). We have used that system systematically to identify new elements of CP formation. In principle, interference with CP

formation can result from defective leading edge formation in migrating cells, from defective nucleokinesis, or from interference with reelin signaling. We have carried out the screening of a chemical library of 2000 molecules ("Diversity Set", provided by the National Cancer Institute, NCI), and identified eleven new molecules that interfere with migration in a manner that remains to be defined. The molecules are active in vitro but not in vivo, presumably because they do not cross the placental barrier and/or are degraded rapidly in vivo. Each of them corresponds to a potential target implicated in the regulation of neuronal migration and cortical development, and requires further study with development of chemical series of analogs. A publication on these results is submitted (Zhou et al., 2006).

3. The role of the seven pass cadherin Celsr3 in cortical development

In Drosophila, the seven pass cadherin Flamingo is implicated in the control of axonal development, dendritic deployment and epithelial planar cell polarity. Mammals have three Flamingo orthologs named Celsr1-3, that are expressed preferentially in the brain, with distinct patterns (Tissir et al., 2002c; Tissir et al., 2002b). Celsr1 is expressed in zones of neural precursor proliferation and plays probably a role in the neuroepithelium, possibly by controlling planar polarity. Celsr2 is expressed in both the neuroepithelium and postmigratory neurons, and remains expressed in the adult brain. Celsr3 expression is restricted to postmigratory neurons, and is particularly high in the forebrain, including the CP.

We inactivated Celsr3 in mice (collaboration between F. Tissir and I. Bar, FUNDP, Namur). The mice die shortly after birth of central ventilation failure. Their forebrain is highly abnormal, with no thalamocortical or corticostriatal connections, absence of internal capsule and of anterior commissure. They have no medial lemniscus, no corticospinal tract and profuse anomalies of longitudinal tracts in the hindbrain and spinal cord that account for the ventilation failure and neonatal death. The phenotype is identical to that generated by inactivation of frizzled-3 (Fzd3), one of the ten mouse frizzled genes (Wang et al. 2003). These results are published (Tissir et al., 2005). We are making a conditional mutant mouse in which the region deleted in the Celsr3 -/- mice is flanked with loxP sites. We have obtained heterozygous animals and should be able to know whether homozygous mutants are viable in a few weeks. In parallel, we have started to develop reagents for cell biological and biochemical studies of the Celsr3 protein. Finally, we have studies by in situ hybridization the expression of several murine orthologs of drosophila PCP genes, in order to assess whether some of them are in a position to interact with Celsr3, and conclude that Dvl3, Vangl2 may be interesting in that context (Tissir and Goffinet, 2006).

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The functional interaction between dorsal and ventral visual stream areas during 3D object discrimination and grasping.

We investigated the shape selectivity of individual neurons in the posterior parietal area LIP of awake behaving rhesus monkeys. Two single-cell studies were started that will provide valuable information for the investigation of the functional interactions between inferior temporal and posterior parietal cortex.

In a first series of experiments, LIP neurons were tested with simple, two-dimensional (2D) shapes during passive fixation. The shapes varied along one dimension, known to be important for grasping. The dimensions tested were curvature along the vertical boundaries, curvature along the horizontal boundaries, tapering in the vertical and in the horizontal direction, orientation, aspect ratio and center-of-mass (Figure 1). Two different sizes were used, 5 degrees and 1 degree. In addition, all neurons were tested in a saccade task, in which the animal had to make an eye movement to a green target after a blue stimulus (the go-signal) dimmed. We searched for responsive neurons during eye movements and during passive fixation while the 2D shapes were presented in the center of the display. Most neurons in the anterior part of LIP (aLIP) were selective for 2D shape: 34 out of 40 neurons tested (85%) showed significant response differences during the presentation of the 2D shapes (example neurons in Figure 2). Surprisingly, many neurons (23/40, 57%) were not responsive during eye movements but very shape selective when the stimuli were presented at the fovea. Another population of neurons (17/40, 43%) did show some modulation during the saccade task and was shape selective when the stimuli were presented in the (peripheral) receptive field of the neuron. We tested neurons with their preferred shape at different positions in the visual field and observed selectivity invariance up to 7.5 degrees eccentricity (for neurons with a foveal receptive field).

A second study investigates the selectivity of posterior parietal neurons for three-dimensional (3D) shapes, in which depth is defined by gradients of binocular disparity. We recently started to record single neurons in the anterior part of LIP and observed strong 3D shape selectivity that can not be accounted for by the responses to the monocular images. Some neurons even preserved their selectivity over a number of positions-in-depth, indicating that these neurons are responding to the gradient of disparity and not just to differences in the position-in-depth of the stimulus.

Finally, we prepared two rhesus monkeys for recording and inactivation studies. These animals will be trained on a 3D-shape discrimination task before recordings will be made in posterior parietal and inferior temporal cortex. Inactivations during these recordings will allow to assess the contribution of each area to 3D shape discrimination.

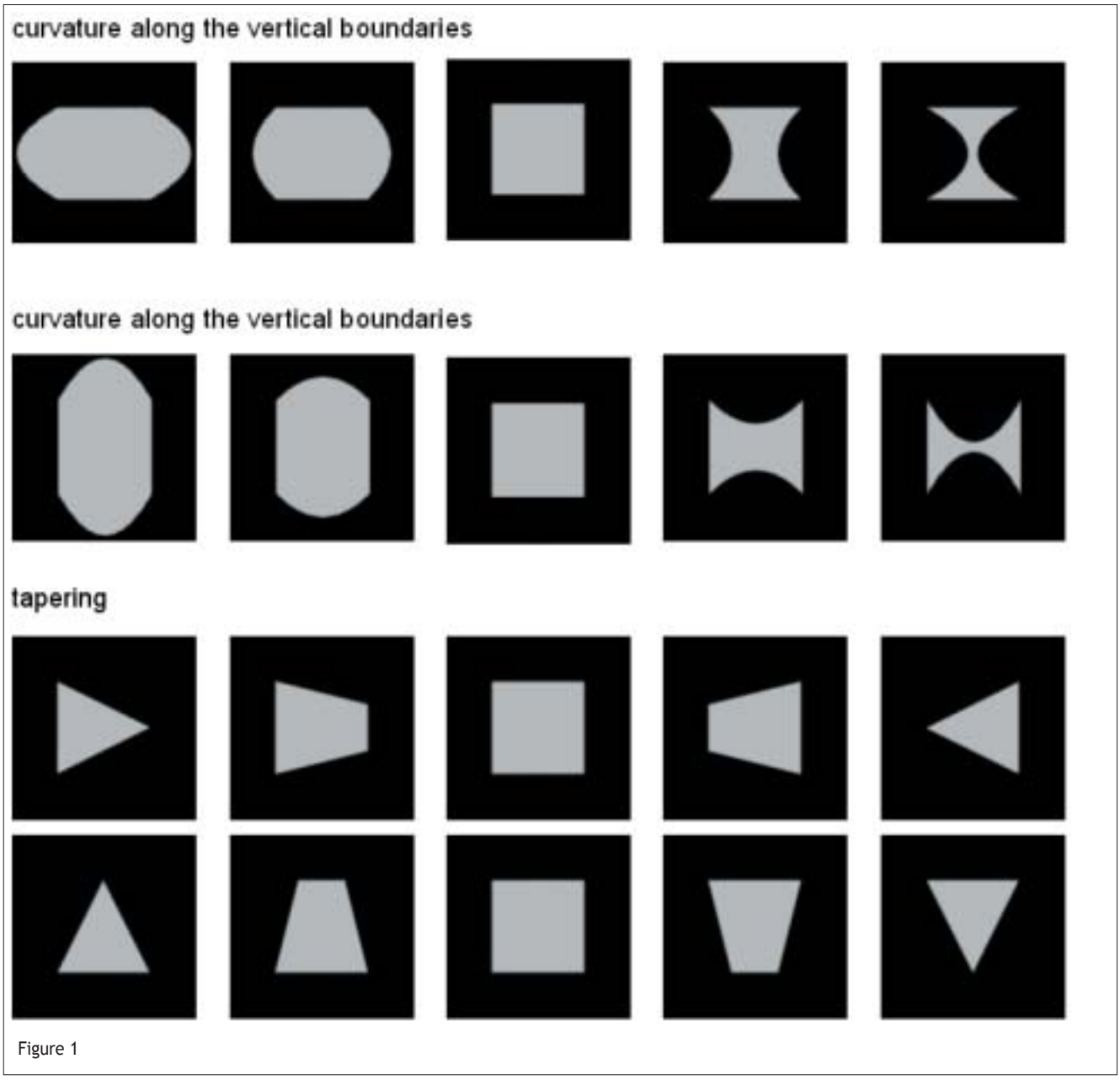
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Figure 1

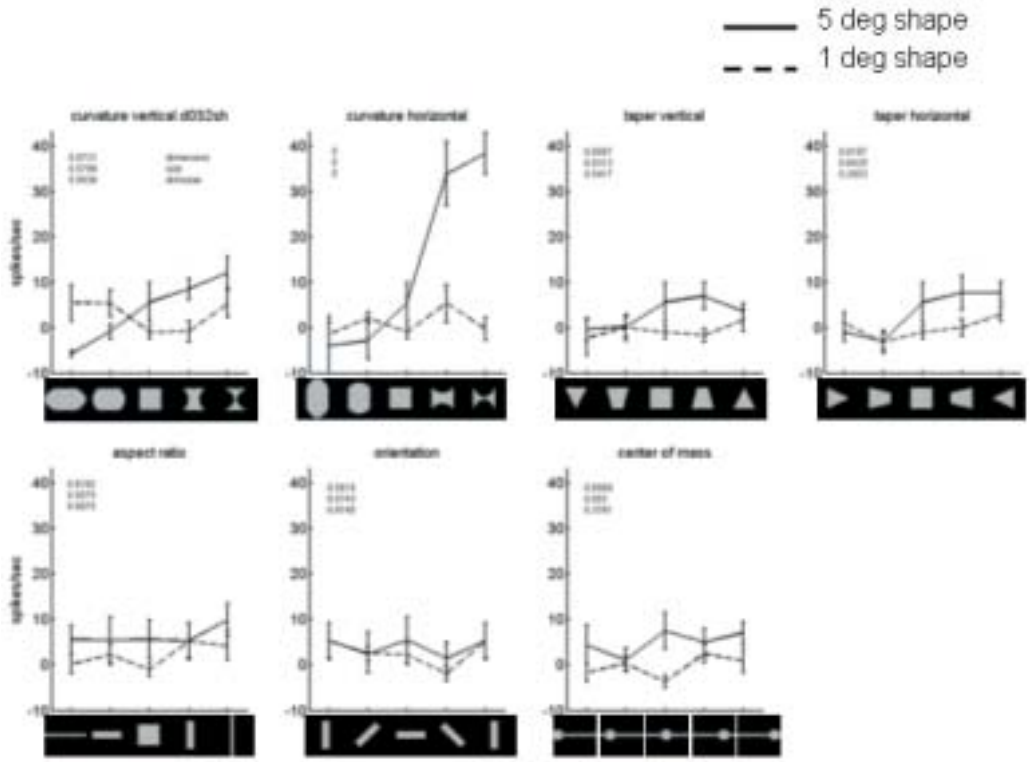
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Figure 2

Progress Report of the Research Group of

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Human Brain Function in Sleep.

Studies in Man by Multimodal Functional Neuroimaging

1. Introduction

Our project aims (i) at further characterizing the neural correlates of human sleep and (ii) at specifying the functional relevance of sleep processes for waking brain function and cognition, at the systems levels, using combined EEG/fMRI acquisitions.

2. Neural correlates of human sleep

In a first stage, this project aims at characterizing the hemodynamic brain responses to neurophysiological transients (spindles, K complexes, slow waves) or oscillations (delta) in NREM sleep.

1. Ballisto-cardiographic artefact rejection method

EEG recording during fMRI acquisition remains a technological challenge. Large artifacts are induced by fMRI acquisitions, especially because of the gradient switching of EPI sequences. Standard adaptive average subtraction has proved efficient in cleaning the EEG recordings from this type of artifact.

Another artifact is due to the movement of the subjects and the electrodes within the magnetic field, due to the ballisto-cardiographic stroke. Although many solutions have been published to reject this artifact, none of them were completely satisfactory for NREM sleep studies. This is due to the fact that the EEG in this stage of sleep is characterized by large amplitude oscillations within the frequency range of the heart rate.

We spend a large part of our research effort in designing a new method, based on independent analysis, which seems to be much more efficient than the available methods.

Further testing and refinement of this new technique are underway.

2. Sleep studies

Our aim was to be able to scan subjects without sleep depriving them beforehand. After an semi-structured interview, the rest/activity rhythm of the subjects is followed for several days. If the rhythm is proved regular, the subjects are scanned between midnight and 4.00 am.

A total of 26 subjects have been scanned so far. We obtained stable NREM sleep, including stage 3 and 4 sleep in 16 subjects. This is an achievement, given that the acquisitions were begun only last June.

Data analysis are underway. Preliminary results show, on a limited number of analyzed subjects, consistent patterns of BOLD responses in relation to anterior and posterior spindles as well as slow waves.

3. Influence of post-training wakefulness, sleep and lack of sleep on learning and memory

Our aim is (i) to characterize the processing of the memory trace during the post-training waking period and (ii) to characterize the effects of sleep deprivation on the sleep-related memory processing

The typical experimental design in this subproject will be to expose the participants to an (explicit or implicit) memory task and retest them on the same task after various delays during the immediate post-training waking period, after one night of sleep or after sleep deprivation. In some cases, both total and partial sleep deprivation will be considered.

1. Effect of sleep and lack of sleep in spatial memory

Sleep takes part in the consolidation of recently acquired memories and their progressive integration into long term stores within cerebral networks. Sleep deprivation, especially on the first post-training night, should hinder these processes. Using functional magnetic resonance imaging (fMRI), we mapped regional cerebral activity during place-finding navigation in a virtual town, immediately after learning and three days later, in subjects either allowed to sleep or totally sleep-deprived on the first post-training night. Results show that covert reorganization of spatial learning-related networks develops during sleep, even when overt performance is not affected by sleep deprivation. At immediate and delayed retrieval, behavioral performance was equal between groups. Place-finding navigation elicited increased brain activity in an extended hippocampo-neocortical network in all subjects. However, in subjects allowed to sleep on the first posttraining night, striatal navigation-related activity increased more than in sleep deprived subjects. Correlation between striatal response and behavioral performance as well as functional connectivity between the striatum and the hippocampus were strengthened in subjects sleeping on the fist post-training night but not in participants sleep deprived on the same night. These data show that brain activity is restructured during sleep in such a way that navigation, initially related to a hippocampus-dependent response pattern, progressively relies on a striatum-dependent network. Both types of neural responses eventually relate to equivalent performance levels, indicating that covert reorganization of brain patterns underlying navigation following sleep is not necessarily accompanied by overt changes in behavior.

2. Offline processing of motor memory

Motor skill learning is a dynamic process that continues covertly after training has ended and eventually leads to delayed increments in performance1. With the finger-tapping task, it is thought that little improvement can occur during post-training wakefulness, a significant gain in performance being observed only after sleep[2-5]. Here, we show that performance temporarily improves early on, 5 to 30 minutes after training. Although this early boost in performance is no longer present 4 hours later, it is predictive of performance levels eventually achieved 48 hours later in the absence of any further practice. Low-frequency repetitive transcranial magnetic stimulation (rTMS) applied over the primary motor cortex (M1) immediately after learning depresses this early boost in performance but does not affect the delayed improvement observed 48 hours later. These results recast our understanding on consolidation

of motor memories in humans, sorting out an early but short-lived boost in performance from the genuine enduring consolidation process.

3. Offline processing of oculomotor memory

Studies of manual and digital sequence learning indicate that motor memories continue to be processed after training has ended, following a succession of identifiable steps. However, it is not known whether this offline memory processing constitutes a basic feature of motor learning and generalizes to the implicit learning of a sequence of eye movements. To assess this hypothesis, we have created the serial oculomotor reaction time task (SORT). Participants were trained to the SORT then tested after either 30 minutes, 5 hours or 24 hours. During training, ocular reaction times decreased monotonically over practice of a repeated sequence, then increased when a different sequence was displayed, demonstrating oculomotor learning of the trained sequence. When tested 30 minutes and 24 hours after training, a significant gain in oculomotor performance was observed, which was no longer present after 5 hours. A gain in performance specific to the learned sequence emerged only 24 hours after training. After testing, a generation task confirmed that most subjects learned implicitly the regularities of the sequence. Our results show that, as for manual or digital sequences, oculomotor sequences can be implicitly learned. The offline processing of oculomotor memories follows distinct stages in a way similar to those observed after manual or digital sequence learning.

4. Offline processing of spatial and procedural memories during the immediate post-training wakefulness

Much remains to be discovered about the fate of recent memories in the human brain. Several studies have reported the reactivation of learning-related cerebral activity during posttraining sleep, suggesting that sleep plays a role in the offline processing and consolidation of memory. However, little is known about how new information is maintained and processed during posttraining wakefulness before sleep, while the brain is actively engaged in other cognitive activities.

We show using fMRI that brain activity elicited during a new learning episode modulates brain responses to an unrelated cognitive task during the awake period that follows the end of practice. This posttraining activity evolves in learning-related cerebral structures, in which functional connections with other brain regions are gradually established or reinforced, and correlates with behavioral performance, following a different time course for hippocampus-dependent and hippocampus-independent memories.

Our experimental approach allowed to characterize the offline evolution of the cerebral correlates of recent memories, without the confounding effect of concurrent practice of the learned material. Results indicate that the human brain extensively processes recent memories already during the first hours of posttraining wakefulness, even when currently coping with unrelated cognitive demands.

These results are under the press in the **Public Library of Science Biology (PloS Biology)**.

4. Non Image Forming (NIF) brain responses to light

Light enhances both alertness and performance in humans. These effects do not correspond to classical visual responses to light but represent Non-Image Forming (NIF) responses, which involve a recently described system of photoreception. Light also profoundly influences regional brain function, beyond classical visual responses. Many studies focused on night time light exposure, but little is known about the daytime responses, especially their neural correlates and temporal dynamics.

Using fMRI, we characterized the neural correlates of the alerting effect of daytime light by assessing the responses to an auditory oddball task before and after exposure to a bright polychromatic white light. Light-induced improvement in subjective alertness was linearly related to an increased responsiveness in the posterior thalamus. In addition, light enhanced responses in a set of cortical areas involved in the oddball paradigm, preventing decreases of activity otherwise observed during continuous darkness. Importantly, the increases in response declined within minutes after the end of the light stimulus, following various regionally-specific dynamics. These findings suggest that light can modulate the activity of subcortical structures involved in alertness, thereby dynamically promoting cortical activity in networks involved in ongoing non-visual cognitive processes.

Progress Report of the Research Group of

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In vitro and *in vivo* studies on the role of the IRAP enzyme/AT_4 receptor system in learning and memory processes

I. INTRODUCTION

1. Ang IV and the IRAP enzyme /AT_4 receptor system

The renin-angiotensin system (RAS) is widely recognised as the most powerful signalling system for controlling sodium balance, body fluid volumes and arterial blood pressure. The major RAS peptide is the octapeptide angiotensin II (Ang II). It is formed by enzymatic processing of Ang I by the angiotensin-converting enzyme (ACE) present in plasma as well as in tissues such as brain, kidney and heart. Although Ang II has long been considered to represent the RAS end product, this system comprises additional peptides with particular physiological functions. In this respect, shorter Ang II fragments such as Ang-(1-7), Ang III and Ang IV are formed via the activity of ACE and other peptidases. The hexapeptide Ang IV sparked great interest because of its wide range of physiological effects. Among those, its facilitatory role in memory acquisition and retrieval is of potential therapeutic interest.

Ang IV binds to AT_1 and AT_2 receptors but only with low affinity. Yet, most of its physiological effects are already observed at nanomolar concentrations and classical non-peptide AT_1 and AT_2 antagonists do not block these effects. This, together with the discovery of high affinity binding sites for [^{125}I]-Ang IV in the central nervous, vascular and renal systems (1-3) led to the concept of a novel angiotensin receptor subtype: the "AT_4 receptor" (4,5). The pharmacological profile of the AT_4 receptor deviates significantly from AT_1 and AT_2 receptors. Instead, it is activated by Ang IV and by more stable synthetic peptide analogues like Norleucine^{l}-Ang IV (Nle^{l}-Ang IV) (6) and Norleucinal (7). These putative AT_4 receptors also constitute cellular targets for hemorphins, a class of endogenous CNS peptides obtained by hydrolysis of the beta chain of hemoglobin (8).

The "AT_4 receptors" have recently been identified as insulin-regulated aminopeptidase (IRAP) or cystinyl aminopeptidase (EC 3.4.11.3), also known as placental leucine aminopeptidase (P-LAP) and oxytocinase (Otase) (7). IRAP is a type II integral membrane protein homologous to aminopeptidase A (AP-A), aminopeptidase N (AP-N), and other Zn^{2+}-dependent enzymes of the gluzincin aminopeptidase family (9, for review see 10). Its different denominations are related to its independent "discovery" by several research teams as well as to differences in the physiological context in which this enzyme was investigated. In insulin-responsive cells, IRAP co-localises with the insulin-dependent glucose transporter GLUT4 in specific intracellular vesicles (11).

2. Role of Ang IV in memory and learning

Initial interest in Ang IV originated from its ability to increase memory recall and learning in passive and conditioned avoidance response studies (12-17). Intracerebroventricular (i.c.v.) administration of the AT_4 agonist Nle^{l}-Ang IV facilitated the rate of acquisition to solve a spatial learning task in the circular water maze, an effect that was blocked by the putative "AT_4 antagonist" Divalinal-Ang IV (18). This ligand also counteracted scopolamine-induced disruption

of spatial learning (19), suggesting an Ang IV-acetylcholine interaction. Electrophysiological and biochemical studies revealed that the cognitive effects of AT_4 agonists are mediated via the hippocampus. Ang IV and its analogues significantly enhanced hippocampal long-term potentiation (LTP) in the dentate gyrus and the CA1 field, both *in vitro* (20) and *in vivo* (21). Ang IV and LVV-hemorphin-7 facilitated potassium-evoked acetylcholine release from rat hippocampal slices (22) and i.c.v. administration of Ang IV induced c-Fos expression in hippocampal pyramidal cells (23). Moreover, autoradiographic studies revealed that AT_4 binding sites are prominent in brain structures important to cognitive processing, including hippocampus (24).

3. Working hypotheses and specific aims of the project

The above-described intriguing findings imply that Ang IV might be a ligand for the putative AT_4 receptors as well as a competitive inhibitor of IRAP's catalytic activity (11). We will perform a critical evaluation of these working hypotheses that IRAP is indeed the AT_4 receptor, and that the IRAP enzyme/AT_4 receptor system represents the only or major cellular recognition and signalling site for Ang IV in the CNS. This research is of special interest in the field of cognition and it may contribute to our understanding of pathophysiological conditions such as Alzheimer's disease.

Homodimer formation is one of the characteristic features of the membrane-bound M1 metallopeptidase family (25) to which IRAP belongs. As dimers, these enzymes have the potential to convey information across cell membranes in the same way as growth factors and cytokine receptors. In this respect, the structurally related AP-N (EC 3.4.11.2) and dipeptidyl-peptidase IV ectoenzymes have already been shown to mediate intracellular signalling (26, 27). Therefore we will investigate the capability of the IRAP enzyme/AT_4 receptor system, after binding with Ang IV, to trigger intracellular signalling pathways in neuronal cells.

AP-N preferentially cleaves peptides *in vitro* with an N-terminal neutral or basic amino acid such as somatostatin, leu- and met-enkephalin, neurokinine A, lysyl-bradykinin, interleukin 8, tuftsin, thymopentin, Ang III and Ang IV (28). It is of particular interest that AT_4 receptor ligands also inhibit AP-N activity (29). Accordingly, IRAP and AP-N could both be involved in the physiological effects exerted by Ang IV. With both *in vitro* and *in vivo* experiments we will try to quantify the contribution of both IRAP and AP-N in the Ang IV-induced effects.

Despite the fact that Ang IV appears to play a role in memory acquisition and learning, there are no *in vivo* data demonstrating the modulation of neurotransmitter release by Ang IV. Therefore, *in vivo* microdialysis will be used to unravel possible effects of Ang IV on the extracellular levels of acetylcholine, glutamate and other neurotransmitters in brain regions involved in learning and memory processes. These experiments will be performed in rats, wild-type mice and IRAP knock-out mice to give decisive answer whether the effects of Ang IV are elicited only by interaction with the IRAP/AT4 system.

Since the major *in vitro* substrates of the IRAP enzyme, oxytocin, vasopressin and somatostatin, are known to play an important role in cognitive function (30-32), it was proposed that Ang IV and other AT_4 receptor ligands could mediate at least part of their

physiological effects by inhibiting IRAP's enzymatic activity (7). In this project, we will investigate if inhibition of IRAP by Ang IV will indeed result in an in vivo release of these neuropeptides of interest. Moreover, selective oxytocin, vasopressin and somatostatin receptor antagonists will be used to block physiological effects induced by Ang IV or its analogues.

These new concepts offer a wide range of original opportunities for examining the physiological roles of the "IRAP/AT_4" and the AP-N systems as well as the mechanisms of action of Ang IV within learning and memory processes.

II. REPORT 2005

1. Exploring the presence and function of the IRAP enzyme/AT4 receptor system in vitro

The fact that both **IRAP and AP-N** could be cellular targets for Ang IV prompted us to compare the ligand interaction properties of recombinant human IRAP and human AP-N by means of [^{125}I]Ang IV binding and catalytic activity measurements. Both enzymes displayed distinct pharmacological profiles. Although both Ang IV and LVV-H7 inhibited their activity, they are more potent IRAP-inhibitors. On the other hand, substance P and L-methionine have a higher potency for AP-N. High affinity binding of [^{125}I]Ang IV occurs to IRAP but only in the presence of the chelators 1,10-phenanthroline and EDTA. In contrast, no high affinity [^{125}I]-Ang IV binding could be detected for AP-N in the absence or presence of chelators. These differences were used to explore for the presence of IRAP and/or AP-N in different cell lines (CHO-K1, COS-7, HEK293, SK-N-MC and MDBK). We provide evidence that mainly IRAP is present in these cell lines and that CHO-K1 cells display the highest level of this enzyme (33).

IRAP and AP-N have one Zn^{2+} binding motif and are member of the M1 aminopeptidase family. Since high affinity [^{125}I]-Ang IV binding to IRAP was only perceptible in the presence of the chelators 1,10-phenanthroline and EDTA, we studied the ion modulation of IRAP and AP-N in greater detail. The catalytic activity of IRAP was studied in membranes of CHO-K1 cells and the catalytic activity of AP-N was studied in membranes of HEK293 cells transfected with human AP-N and purified soluble porcine kidney AP-N. Both enzymes behaved similarly:

- Planar divalent cation chelators like 1,10-phenanthroline produced a complete and concentration-dependent inhibition of the activity of both enzymes, albeit with low potency and with Hill slopes >1.
- In contrast, acetic acid containing chelators like EDTA only weakly affected the activity of both enzymes, but they increased the potency of the planar chelators till a limit, at which Hill slopes became close to unity.
- Moreover, competition between 1,10-phenanthroline and the substrate only took place in the presence of EDTA.
- Finally, the inhibitory effect of EDTA plus 1,10-phenanthroline could be completely reversed by Zn^{2+}. The latter effect was potentiated when Ca^{2+} was added to the incubation medium.

These findings are new and suggest that EDTA is capable of removing a modulatory ion from an allosteric site at both enzymes, facilitating the direct interaction between 1,10-phenanthroline and the catalytic Zn^{2+}. However, an important distinction between both apo-enzymes (i.e. enzymes lacking the catalytic Zn^{2+}) is that high affinity [^{125}I]-Ang IV binding only takes place to apo-IRAP. The selectivity of the process greatly facilitates the detection and quantification of **IRAP** and this opens new possibilities for the study of its structural and functional properties under normal and pathophysiological conditions (34, 35).

In order to investigate whether inhibition of **IRAP and/or AP-N** is involved in the effect of Ang IV in the rat striatum, we performed a combined *in vitro/in vivo* study. The striatum, a brain nucleus involved in the control of movement but also in learning and memory processes (36), contains both IRAP (37) and AP-N (38). In striatal membranes, by using the AP-N selective inhibitor 7B, we demonstrated that about 60% of the aminopeptidase activity could be attributed to AP-N (pK_i = 9.20). Higher concentrations of 7B were capable of inhibiting IRAP as well (pK_i = 7.26) (39).

We have also started to investigate whether IRAP/AT4 can trigger certain **intracellular signalling pathways** after binding of Ang IV and related ligands. For this purpose we have already set up assays to measure intracellular Ca^{2+} concentration and [^{3}H]-thymidine incorporation (to measure cell division) in cells that express IRAP/AT_4. In collaboration with the Vascular and Renal research laboratory of M. Ortega in Madrid, Ang IV was found to regulate the expression of NF-κB related genes in vascular smooth muscle cells. These genes are known to play a role in inflammatory responses. By measuring the [^{125}I]-Ang IV binding and enzyme activity we provided evidence for the presence of IRAP/AT_4 in these cells, suggesting its possible role in these processes (40). It is not yet clear whether Ang IV also regulates these genes in neuronal cells.

2. Investigation of the involvement and function of the IRAP /AT_4 system in vivo

To quantify the memory-promoting effects of Ang IV and several other AT_4 receptor ligands (e.g. Norleucinal, LVV-hemorphin-7) within this project, the introduction of **behavioural tests for memory and learning** in our laboratory was necessary. In this context, we have introduced and validated a complete set-up of the well-established 'Morris water maze' for spatial memory (41) with a Noldus EthoVision video tracking system. The Morris water maze is the most widely used technique for the assessment of spatial learning and memory in mice and rats, in which the animal subjects learn to locate a platform hidden under the water surface of a circular swimming pool. Spatial learning and memory of Wistar Hannover, Sprague Dawley and Agouti Brown rats was evaluated under different experimental conditions. The testing room and training protocol were configured to obtain a robust spatial strategy in the animal subjects. Since no differences could be demonstrated between the different strains, Wistar Hannover rats were selected for further experiments. This rat strain is also used for the microdialysis experiments in the laboratory. In preliminary experiments within the Morris water maze, i.c.v. injection of 1 nmol Ang IV had no effects compared to controls with i.c.v. injection of Ringer's solution. This result confirms previous experiments where a single i.c.v. injection of 1 nmol Ang IV (42) or daily repeated i.c.v. injection of 1 nmol Nle^1-Ang IV (43) had no effect compared to

the controls. However, infusion of Nleu^1-Ang IV (18) or daily repeated injection of Nleu^1-Ang IV (19, 43) had a clear learning and memory enhancing effect in rat models for cognitive dysfunction. Therefore we will introduce rat models for cognitive dysfunction based on i.c.v. injection of scopolamine and mecylamine (43) and i.c.v. infusion of amyloid-ß(1-42) (44). The latter model will possibly give a lead towards the effect of Ang IV in the pathofysiology of Alzheimer's disease. In a later stadium we are planning behavioural experiments in mouse models for Alzheimer's disease and experiments with IRAP knock-out mice.

We previously demonstrated that local administration of Ang IV via a microdialysis probe in the rat striatum caused a dose-dependent increase in extracellular dopamine (DA) levels, which was not AT_1 or AT_2 receptor dependent (45). As part of the combined *in vitro*/*in vivo* study (see also point II.1.) to investigate whether inhibition of **IRAP and/or AP-N** is involved in this effect of Ang IV in the **striatum** of the rat, we found that *in vivo* neither inhibition of IRAP nor AP-N activity appeared to be involved in this Ang IV-mediated effect in the striatum (39). Indeed, local administered 7B in concentrations at which it inhibits AP-N as well as IRAP was not capable of producing a DA release such as observed with Ang IV alone. However, co-administration in the striatum of 7B at a concentration selective for AP-N together with Ang IV elicited a higher increase of the extracellular DA concentration as compared to Ang IV alone. This is probably due to an inhibition of AP-N by 7B, thereby lengthening the half-life of Ang IV, which is only about 10 seconds in a normal in vivo environment (46). Indeed, AP-N is implied in the degradation of Ang IV (47). On the other hand, co-administration of 7B in a concentration at which it inhibits both AP-N and IRAP completely abolished the effect of Ang IV. Possibly, 7B interacts with the binding of Ang IV at AP-N and/or IRAP, resulting in the abolishment of the Ang IV-induced effect. The AP-N selective inhibitor 7B would then behave as an antagonist of AP-N and/or IRAP, thereby inhibiting the binding of Ang IV but not causing an effect itself. We therefore hypothesize that the effect of Ang IV on DA release in the striatum is mediated via activation of IRAP and/or AP-N, now acting as receptors for Ang IV.

Since hippocampal cholinergic-glutamatergic interactions are important for learning and memory (48), AT_4/IRAP-mediated effects might influence **cholinergic and glutamatergic neurotransmission in hippocampus**. Besides clinical evidence obtained with cholinesterase inhibitors and memantine, it is also widely known that **GABA** is implicated in memory function, e.g. retrograde amnesia in patients taking benzodiazepines or significant improvement of water maze performance in transgenic mice lacking the α5 $GABA_A$ receptor subunit (49). A meta-analysis based on studies of four behavioural tasks of learning and memory (Morris water maze, radial maze, passive avoidance and spontaneous alternation) demonstrated that also the **monoaminergic** (DA, serotonin (5-HT), noradrenalin) systems are involved in cognitive processing (50). To our knowledge, there are at present no *in vivo* data concerning Ang IV-mediated modulation of hippocampal neurotransmitter release available in literature.

- Therefore, we investigated the effects of different doses of Ang IV administered via an i.c.v. injection on the extracellular levels of **glutamate, GABA, DA and 5-HT** in rats and mice. Validated microbore liquid chromatography (LC) systems running for routine analysis of monoamine and amino acid dialysate concentrations are available in our laboratory. Using in

vivo microdialysis in mice, we report that an i.c.v. injection of Ang IV (10 nmol/2µL) reduced the extracellular levels of GABA without changing the levels of glutamate in the hippocampus (51). We propose that this decrease in hippocampal GABAergic activity is part of the memory enhancing effect displayed by Ang IV. We also showed that i.c.v. administered Ang IV caused an increase in the extracellular DA and 5-HT concentrations in the hippocampus of the rat (52). More experiments with selective DA and 5-HT receptor antagonists might shed a light on the possible involvement of these hippocampal monoamine increases in spatial memory.

- To be able to study the effects of i.c.v. Ang IV administrations on the extracellular **acetylcholine (Ach)** concentrations, we first had to introduce and validate an analytical microbore LC set-up with electrochemical detection. This method is based on ion-pair chromatographic separation of endogenous cholines, oxidation of ACh to H_2O_2 and subsequent amperometric detection on a peroxidase-coated glassy-carbon electrode (53, 54). The feasibility of this method for the determination of basal ACh in brain microdialysates without the use of acetylcholinesterase inhibitors has been demonstrated in literature but the method has only been superficially validated and little is known about its use in routine analysis. Therefore the chromatographic parameters of the method for determination of ACh in brain microdialysates were studied. After selection of appropriate chromatographic conditions our method was proven to be linear, repeatable and reproducible. The limited robustness of the method was characterized by deterioration of the used enzyme systems and the chromatographic column. A rapid sensitivity decrease of approximately 10% per day was earlier reported and attributed to the degradation of the peroxidase coating (55). We obtained a sensitivity decrease of approximately 1% per day by amelioration of the coating protocol. The mobile phases reported for this method are typically alkaline phosphate buffers without organic modifiers (53). These conditions rapidly degrade chromatographic columns. However Sotoyama et al. (54) reported a mobile phase at pH 6.5 could be used and we demonstrated that the pH of the mobile phase does not influence the sensitivity of the method within the range 6.5 to 8.5. Therefore we argue that a mobile phase with pH 6.5 should be used to improve the column stability. The developed method has been applied in preliminary microdialysis experiments with i.c.v. administration of Ang IV. A decrease in extracellular hippocampal ACh concentrations was observed after i.c.v. injection of 1 nmol and 10 nmol Ang IV. Further experiments are planned to study the alterations of the cholinergic system in rat models for cognitive dysfunction and to evaluate the role of the observed modulation of the cholinergic system in the cognitive effects of Ang IV.

An important hypothesis states that **Ang IV mediates its memory-enhancing effects by preventing IRAP-mediated degradation of other neuropeptides involved in memory functioning** (7). As stated above, **vasopressin, somatostatin and oxytocin** are known substrates for IRAP and are involved in cognitive functioning (30-32). It is tempting to speculate that inhibition of IRAP's catalytic activity by Ang IV may enhance the activity of these neuropeptides.

- Therefore, it will be of major interest within this project to **monitor neuropeptide release** in brain dialysates following administration of Ang IV or its analogues. If neuropeptides are

administered via a microdialysis probe, their extracellular concentrations are only a reflection of the absolute concentrations in the perfusion fluid. Therefore the relative loss (RL) of the peptides of interest has to be determined. Currently, we are investigating the in vitro RL of Ang IV (in a concentration of 10 µM) and studying enhancement of the RL of this peptide by addition of e.g. bovine serum albumine and triton X-100 to the perfusion medium. In parallel, we are developing a microbore LC method with UV-detection for the separation of Ang II and 5 of its metabolites (Ang III, Ang IV, Ang-(1-7), Ang-(3-7) and Ang-(4-8) in microdialysates. The influence of several parameters on the separation and UV-sensitivity is investigated. Since, the extracellular concentrations of neuropeptides are extremely low (1-100 pM), a very sensitive analytical technique is required for their quantification in microdialysates. Therefore, we are developing a column switching-nano LC-MS/MS method for the determination of the Ang IV concentrations in hippocampal microdialysates.

- We obtained data in favour of the hypothesis that Ang IV facilitates memory by preventing IRAP-mediated degradation of **vasopressin** (51). Indeed, we showed that the above-described Ang IV-induced decrease in mice hippocampal GABA levels is mediated by the **vasopressin V1a receptor**. Accordingly, i.c.v. injection of vasopressin in mice reduced the extracellular hippocampal levels of GABA but not glutamate. The attenuation of baseline GABA release exerted by both Ang IV and vasopressin were clearly reversed by blocking the vasopressin V1a receptor with the specific non-peptidergic V1a receptor antagonist SR49059. These results suggest that the effect of Ang IV on hippocampal GABA is mediated through the blockade of IRAP's catalytic site and the consequent increase of vasopressin levels. The vasopressin V1a receptor antagonist will be tested in a further stage of this project on its ability to block the memory-enhancing effects of Ang IV within the Morris water maze task.

- As described above, i.c.v. administered Ang IV increased extracellular hippocampal DA and 5-HT levels in the rat. It was already shown that increasing the DA and 5-HT concentrations in the hippocampus is anticonvulsant in the acute pilocarpine rat seizure model (56). In agreement, we showed that i.c.v. administered Ang IV protected rats against pilocarpine-induced seizures. The Ang IV-mediated anticonvulsant effect and increases in DA and 5-HT release were not AT_1 receptor dependent but could be blocked by concomitant i.c.v. administration of the somatostatin receptor 2 antagonist cyanamid 154806. This suggests that Ang IV can induce **somatostatin 2 receptor** mediated effects. **Somatostatin-14** possesses anticonvulsant properties (57) via the somatostatin 2 receptor (58). We showed that i.c.v. administration of somatostatin-14 causes similar increases in the extracellular hippocampal DA and 5-HT levels as compared to Ang IV and also protected the rats against pilocarpine-induced seizures. Since Ang IV is a competitive inhibitor of the IRAP enzyme, which metabolises somatostatin-14 *in vitro* into the inactive de-[Ala-Gly]-somatostatin-14 fragment (7, 59), we hypothesize that i.c.v. administration of Ang IV causes an increase in the somatostatin-14 concentration in the brain, which then exerts its effects via the somatostatin 2 receptor. Based on these experiments, we will also test the potential of the somatostatin receptor 2 antagonist to block the Ang IV-enhanced performance to solve a Morris water maze task.

III. PUBLICATION LIST OF THE EFAR RESEARCH GROUP ON THE IRAP ENZYME/AT4 RECEPTOR SYSTEM

1. International peer-reviewed publications on this specific topic before 2005

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- Demaegdt H, Vanderheyden P, De Backer JP, Mosselmans S, Laeremans H, Le MT, Kersemans V, Michotte Y and Vauquelin G (2004) Endogenous cystinyl aminopeptidase in Chinese hamster ovary cells: characterization by [(125)I]Ang IV binding and catalytic activity. Biochem Pharmacol 68:885-892. (scientific impact factor = 3.4)
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Phosphorylation of the amyloid precursor protein intracellular domain: regulation of the production of ß-amyloid peptide and transcriptional activity?

Alzheimer disease, the most frequent cause of dementia, is characterized by the presence of typical microscopic lesions in the brain of affected patients. The coexistence of intraneuronal neurofibrillary tangles and extracellular senile plaques allows confirmation of the clinical diagnosis of the disease (1).

Neurofibrillary tangles are made of paired helical filaments (PHFs) containing the microtubule-associated protein, tau (2-4). In Alzheimer disease, tau is hyperphosphorylated, and many serine and threonine residues (5, 6) that are found to be phosphorylated in PHF tau can be phosphorylated by GSK3 both *in vitro* and in transfected cells (7-9).

Senile plaques contain an amyloid core that is mainly constituted of amyloid-β peptide (Aβ) (10), which is derived from the amyloid precursor protein (APP) (11, 12). The APP gene encodes 10 different APP isoforms (13) with an amino acid content varying from 365 to 770 amino acids. The neuronal APP is a single pass type I transmembrane protein containing 695 amino acids (11) that is processed by amyloidogenic and nonamyloidogenic catabolic pathways. The β-cleavage of APP, catalyzed by the well characterized aspartyl protease β-site APP-cleaving enzyme 1 (BACE1) (14), produces a C-terminal fragment of APP (CTF), which is further cleaved by γ-secretase to generate Aβ. The γ-secretase activity is found as a multiprotein complex containing at least four different proteins: Aph-1, nicastrin, presenilin, and Pen-2 (15, 16). APP can also be cleaved within the Aβ sequence by an α-secretase. The α-cleavage of APP generates a soluble N-terminal fragment (sαAPP) and a 83-membrane-anchored C-terminal fragment (C83). Experimental evidence indicates that the α-cleavage of APP695 could be performed by members of the desintegrin and metalloprotease family, ADAM10 and ADAM17 (17). The short intracellular C-terminal domain of APP can be phosphorylated in vitro and in vivo by several protein kinases such as cdk5, c-Jun N-terminal kinase, and GSK3 (18-20).

Another substrate of GSK3 is β-catenin, an essential protein of the Wnt signaling pathway. In the absence of a Wnt ligand, GSK3 activity is not inhibited, resulting in the phosphorylation of soluble β-catenin for ubiquitin-proteasome-mediated degradation (21). Alternatively, as a result of GSK3 inactivation by Wnt signaling, intracellular levels of β-catenin increase, allowing its binding to components of the high mobility group family of transcription factors and its translocation into the nucleus.

LiCl, an inhibitor of GSK3 (22), reduces the phosphorylation of tau in rat cultured neurons (23-25). Interestingly, LiCl was recently reported to decrease Aβ production in both transfected cells and transgenic mice (26-29). Therefore, lithium could be a combined therapeutic agent, inhibiting both the phosphorylation of tau and the production of Aβ.

Thanks to the Queen Elisabeth Medical Foundation, we have studied the effect of LiCl on the production of Aβ by transfected CHO cells and rat cultured neurons expressing human APP695. Following treatment by LiCl, this GSK3 inhibitor decreased the phosphorylation of tau in neurons and induced the nuclear translocation of β-catenin in both CHO cells and neurons. It

was found that LiCl increased the β-secretase activity and consequently increased the amount of CTF generated from human APP695. The cleavage of βCTF by an unchanged γ-secretase activity led to an overproduction of Aβ. SB415286, another GSK3 inhibitor, induced the nuclear translocation of β-catenin and slightly decreased neuronal Aβ production. Taken together, these results clearly demonstrate that LiCl stimulates the amyloidogenic pathway of human APP independently of its inhibition of GSK3.

These results were published in 2005 in the Journal of Biological Chemistry (J. Biol. Chem., Vol. 280, Issue 39, 33220-33227) in a paper entitled " **Lithium Chloride Increases the Production of Amyloid-β Peptide Independently from Its Inhibition of Glycogen Synthase Kinase 3** " by Christine Feyt, Pascal Kienlen-Campard, Karelle Leroy, Francisca N'Kuli, Pierre J. Courtoy, Jean-Pierre Brion, and Jean-Noël Octave.

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Distinct contribution of parietal and frontal cortical areas to the control of finger and hand movements

Background

The aim of the present project was to determine the distinct contribution of the different frontal and parietal motor areas involved in the control of finger and hand movements. Small object manipulation is essential in numerous human activities though its neural bases are still largely unknown. Recent functional imaging studies have shown that precision grasping activates a large bilateral fronto-parietal network, although their respective contribution remains largely unknown. However, if functional brain imaging studies have provided us with an exhaustive picture of the brain regions involved in hand movements, this approach does not allow us to make inferences about the causal relationship between the activity in these regions and the processes under investigation. Transcranial magnetic stimulation (TMS) has proved very useful to overcome this limitation by producing, in healthy subjects, transient virtual lesions of restricted brain areas. Combined with a precise quantification of the deficits resulting from such virtual lesions (Duque et al., 2003), this approach permits to infer the contribution of the stimulated brain area to the task under investigation. In order to ascertain the coil position over the targeted brain area, we used a TMS onto MRI coregistration technique developed in the laboratory (Noirhomme et al., 2004). In addition, individual coordinates of the TMS sites were normalized with respect to the Montreal Neurological Institute (MNI) brain atlas (see Davare et al., under revision).

1. Distinct contribution of parietal and frontal cortical areas to the control of finger and hand movements

The aim of this first study was to was to determine the contribution of the ventral (PMv) and dorsal (PMd) premotor areas in precision grasping. In monkeys, it has been suggested that PMd and PMv are part of two independent circuits, originating from the posterior parietal cortex and controlling, respectively, the reaching and grasping components of goal-directed hand movements. In humans, however, the respective contribution of PMv and PMd to hand movements, the time course of their involvement and their hemispheric dominance are still largely unknown. In order to dissociate the role of PMv and PMd in the control of hand and finger movements, we produced, by means of TMS, transient virtual lesions of these two areas in both hemispheres, in healthy subjects performing a grip-lift task with their right, dominant, hand. We found that a virtual lesion of PMv specifically impaired the grasping component of these movements: a lesion of either the left or right PMv altered the correct positioning of fingers on the object, a prerequisite for an efficient grasping, whereas lesioning the left, contralateral, PMv disturbed the sequential recruitment of intrinsic hand muscles, all other movement parameters being unaffected by a PMv lesion. On the other hand, we found that a virtual lesion of the left PMd impaired the proper coupling between the grasping and lifting phases, as evidenced by the TMS-induced delay in the recruitment of proximal muscles responsible for the lifting phase; lesioning the right PMd failed to affect dominant hand movements.These results provide the first compelling evidence for a neuronal dissociation

between the different phases of precision grasping in human premotor cortex (Davare et al., under revision).

A second study focused on the contribution of the ipsilateral primary motor cortex (iM1) to hand movements. To address this issue, we elicited transient virtual lesions of iM1 by means of TMS in healthy subjects performing either a grip-lift task or a step-tracking task with their right dominant hand. We found that, irrespective of the task, a virtual lesion of iM1 altered the timing of the muscle recruitment. In the grip-lift task, this led to a less coordinated sequence of grip and lift movements and, in the step-tracking task, to a perturbation of the movement trajectory. In the step-tracking task, we have demonstrated that disrupting iM1 activity may, depending on the TMS delay, either advance or delay the muscle recruitment. This study suggests that a critical contribution of iM1 to hand movements is to determine the appropriate timing for muscle recruitment, most likely through either inhibitory or facilitatory transcallosal influences onto the contralateral M1 (cM1). iM1 would therefore contribute to shape precisely the muscular command originating from cM1 (Davare et al., in press).We are currently investigating the role of the PPC in precision grasping.

Finally, we studied the relationship between number processing and finger movements; this issue is very pertinent since functional brain imaging studies have shown that motor-related areas are activated during calculation, suggesting that they may participate in number processing. Further evidence about a possible functional relationship between fingers and numbers comes from developmental studies showing that, in children, finger movements play a crucial role during the acquisition of counting. If the hypothesis that motor-related areas participate in number processing is valid, their actual contribution remains to be identified. To address this issue, we applied TMS over the primary motor cortex in order to monitor corticospinal (CS) excitability changes during various enumeration tasks. We found an increase in CS excitability during dot enumeration, but irrespective of the use of the number series or the alphabet to enumerate items. Similar results were obtained when dots were presented simultaneously or sequentially. Additional control tasks allowed us to rule out that attention allocation or articulation processes were responsible for this increased CS excitability. Because we failed to evidence a specific increase in CS excitability while using numbers to enumerate objects as compared with letters, we suggest that motor-related contribution to enumeration may be to match individual objects with items from any ordered series. (Andres et al., submitted).

2. Central representation of hand movements

Secondly, we investigated hand movement representations in the central nervous system. Indeed, interaction with objects is critical in most daily activities and humans are able to access *a priori* knowledge of an object or of a tool to use it. Clinical studies on the ideomotor apraxia, a deficit characterized by an inability to recall the appropriate hand posture to use tools, have suggested that the left PPC may store a repertoire of gestures necessary to utilize tools. Moreover, several imaging studies have suggested that, within the PPC, the critical region for hand movement representations may be the left inferior parietal lobule. To address

this issue we first developed and validated an original task where subjects were required to perform judgments on object-hand interactions (Pelgrims et al., 2005). We then used that task to investigate the respective contribution of the supramarginalis (SMG) and angular (AG) gyri to hand movement representations remains unknown. To address this issue we used repetitive TMS to induce transient virtual lesions of these two areas in healthy subjects trained to perform that task. The present study suggests that the gesture repertoire necessary to manipulate tools adequately is encoded in the left SMG (Pelgrims et al., submitted).

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Progress Report of the Research Group of

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Characterization of the role of G protein-coupled receptors in the central nervous system by using genetically invalidated mouse models.

1. Overview

The activities of the group are centered ontao G protein-coupled receptors, which represent the largest family among membrane receptors. They all share a common structural organization with seven transmembrane segments, and a common way of modulating cell function through a family of heterotrimeric G proteins. About 180 G protein-coupled receptor types and subtypes have been functionally characterized to date in mammalian species, and about 100 orphan receptors are presently available in the literature or the databases. Orphan receptors potentially constitute elements of unknown communication pathways in various systems. The general aim of this program is to identify novel receptors playing a role in brain physiology. The characterization of the function of known receptors or recently characterized receptors, by using, among other approaches, knockout models, has also been pursued.

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

Prolactin-releasing peptide (PrRP) is a recently described neuropeptide, isolated as the natural ligand of the previously orphan G protein-coupled receptor GPR10. PrRP was named following its initial description as a positive regulator of prolactin hormone release by pituitary lactotrophs, and is now described as a regulator of pituitary hormones secretion and feeding behavior. It is also expressed in brain areas involved in the processing of nociceptive signals. We have generated GPR10-deficient mice, which were fertile and did not display obvious abnormalities, and have characterized previously its role in the control of the stress responses and opiate responses (see previous report). In order to complement the study, we have performed a number of additional experiments.

Previous experiments had shown that GPR10-invalidated mice displayed a higher sensitivity to opiate agonists, suggesting an interaction between the opioid system and GPR10. Central administration of PrRP promoted an hyperalgesia in wild-type mice, and was able to reverse the analgesia induced by morphine in the tail-immersion test. We have now investigated the potential interaction of the PrRP-GPR10 system with previously characterized anti-opioid peptidergic systems, namely NPFF and nociceptin, using the tail-immersion test as readout. Intracerebroventricular administration of nociceptin promoted hyperalgesia and reversed morphine-induced analgesia, without significant differences between wild-type and knockout mice. In our conditions, NPFF did not modify the basal nociceptive threshold of wild-type mice, but antagonized the effects of morphine. Surprisingly, in knockout animals, NPFF had an analgesic effect, and was unable to reverse the effects of morphine. These results indicate that some of the central actions of NPFF require a functional PrRP system, and that inactivation of this system unmasks analgesic properties of NPFF, that likely correspond to the spinal effect described in the literature. It appears therefore that the PrRP-GPR10 system is located downstream of the NPFF-GPR10 system in a common central antiopioid pathway.

In order to investigate whether the relative analgesia observed in knockout mice might be

related to increased proenkephalin expression, we investigated the distribution of proenkephalin transcripts in the brain of both genotypes by in situ hybridization. Similar levels of proenkephalin transcripts were measured in various brain areas of wild-type and knockout animals, including several pain-associated areas such as the parabrachial nucleus, the dorsal raphe nucleus, the bed nucleus of stria terminalis and the central amygdala. A modest upregulation was observed in basal ganglia (caudate-putamen and nucleus accumbens), in which GPR10 is poorly expressed, thus suggesting an indirect consequence of GPR10 inactivation. Using [^{3}H]-DAMGO as radioligand, we also characterized the μ binding sites in a saturation binding assay. Similar K_D and B_{max} values were found on whole brain membranes from wild-type and knockout mice. The δ opioid receptor was also tested using [^{3}H]-Naltrindole as tracer in a binding assay on whole brain membranes, but no difference in K_D or B_{max} were observed. The functional response of the μ and δ receptors was evaluated on whole brain membranes and on brain slices in [^{35}S]GTPγS binding assays. On whole brain membranes, the EC_{50} and E_{max} of the μ receptor, and the EC_{50} and E_{max} of the δ receptor were similar for wild-type and knockout mice. No differences in the binding distribution were detected on brain sections for the μ receptor. Also, the pronociceptin and NPFF transcript levels were evaluated by in-situ hybridization and/or quantitative RT-PCR. Both transcripts were found at similar levels in several brain areas of wild-type and knockout mice. From these experiments, it appears therefore that no gross alteration of the opioid and antiopioid systems in pain-associated areas has occurred as a compensatory mechanism in GPR10 knockout mice.

Altogether, we demonstrated an important role of the PrRP-GPR10 system in the modulation of the various actions of opiates. Knockout mice displayed a higher nociceptive threshold in some settings, increased analgesic and rewarding effects of morphine, increased stress-induced analgesia, reduced tolerance to morphine and a reduction of naloxone-precipitated withdrawal symptoms. Interestingly, naloxone reversed the analgesic effects of morphine, but affected also basal levels of the knockout animals in several tests, suggesting that GPR10 disruption generates or unmasks a basal opioid tone that is not detectable in wild-type animals. In line with these observations, we also observed that intracerebroventricular administration of PrRP results in hyperalgesia and reverses the antinociceptive effects of morphine in wild-type mice. The absence of effects of the peptide in knockout mice demonstrates that GPR10 is the sole target of PrRP in vivo. Altogether, these observations establish the involvement of GPR10 in the modulation of pain signal processing.

The observed properties of the PrRP-GPR10 system are reminiscent of other opioid-modulating systems described previously. These other opioid-modulating systems (also referred to as anti-opioid systems), include the neuropeptides CCK, NPFF, nociceptin and dynorphin, and their respective receptors. Although the biological actions of these various peptides are not superimposable, they were all reported to counteract some of the main behavioral effects of morphine in laboratory animals, including analgesia, tolerance, reward and dependence. Our observations qualify the PrRP-GPR10 system as a new anti-opioid system, in addition to the CCK2, ORL1 and NPFF receptors. They suggests that GPR10 might constitute a new pharmacological target for the clinical management of pain, opioid side-effects and the treatment of addictive disorders (Laurent et al. 2005).

3. Characterization of NPFF receptor antagonists

Neuropeptide FF (NPFF) has been proposed to play a role in pain modulation, opioid tolerance, and several other physiological processes. However, pharmacological agents that would help define physiological roles for this peptide are missing. We have collaborated with the group of Brigitte Kieffer (Strasbourg) and reported the discovery of a potent and selective NPFF receptor antagonist, RF9, that can be administered systemically. This compound does not show any effects by itself but can block efficiently the increase in blood pressure and heart rate evoked by NPFF. When chronically coinjected with heroin, RF9 completely blocks the delayed and long-lasting paradoxical opioid-induced hyperalgesia and prevents the development of associated tolerance. These data indicate that NPFF receptors are part of a bona fide antiopioid system and that selective antagonists of these receptors could represent useful therapeutic agents for improving the efficacy of opioids in chronic pain treatment (Simonin et al. 2005).

4. Characterization of the FPRL2 receptor

The identification of the peptide F2L, derived from the amino-terminal cleavage of the intracellular heme-binding protein HBP, as the ligand of the FPRL2 receptor has been published (Migeotte et al. 2005). To get insight into the biological significance of F2L action by using an animal model, we searched for murine receptors for F2L using receptor-transfected cell lines. Surprisingly, although the murine FPR family has at least 8 members instead of three in human, only one murine receptor, fpr2, responded to human and murine F2L with EC50s ~500 nM in both calcium flux and cAMP inhibition assays. Fpr2 is expressed in multiple leukocytes including neutrophils, dendritic cells and macrophages. The pharmacology of this receptor is presently being studied further (unpublished).

5. Characterization of chemerin and the ChemR23 receptor

We have recently characterized chemerin, a novel chemoattractant protein, which acts through ChemR23 and is abundant in a diverse set of human inflammatory fluids. Chemerin is secreted as a precursor of low biological activity, which upon proteolytic cleavage of its COOH-terminal domain, is converted into a potent and highly specific agonist of ChemR23.

Using monoclonal antibodies and functional assays, we have shown that blood plasmacytoid and myeloid DCs express functional ChemR23. Recombinant chemerin induced the transmigration of plasmacytoid and myeloid DCs across an endothelial cell monolayer. In secondary lymphoid organs (lymph nodes and tonsils), ChemR23 is expressed by $CD123^+$ plasmacytoid DCs and by $CD1a^+$ DC-$SIGN^+$ DCs in the interfollicular T cell area. $ChemR23^+$ DCs were also observed in dermis from normal skin, whereas Langerhans cells were negative. Chemerin expression was selectively detected on the luminal side of high endothelial venules in secondary lymphoid organs and in dermal endothelial vessels of lupus erythematosus skin lesions. $Chemerin^+$ endothelial cells were surrounded by $ChemR23^+$ plasmacytoid DCs. Thus, ChemR23 is expressed and functional in plasmacytoid DCs, a property shared only by CXCR4 among chemotactic receptors. This finding, together with the selective expression of the cognate ligand on the luminal side of high endothelial venules and inflamed endothelium, suggests a key role of the ChemR23/chemerin axis in directing plasmacytoid DC trafficking (Vermi et al. 2005).

Dendritic cells and macrophages are professional antigen-presenting cells (APC) that play a

central role in initiating immune responses, linking innate and adaptive immunity. Given the fact that APCs are often preceded by polymorphonuclear cells (PMN) in inflammatory infiltrates, we hypothesized that PMN could mediate chemerin generation. We have demonstrated that human degranulated PMNs release proteases that efficiently convert prochemerin into active chemerin. The use of specific protease inhibitors allowed us to identify the neutrophil serine proteases cathepsin G and elastase as responsible for this process. Mass spectrometry analysis of processed prochemerin showed that each protease generates specifically a distinct form of active chemerin, differing in their C terminus and initially identified in human inflammatory fluids. These findings strongly suggest that bioactive chemerin generation takes place during the early stages of inflammation, underscoring the functional contribution of chemerin as a bridge between innate and adaptive immunity (Wittamer et al. 2005).

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

Adenosine is released from metabolically active cells or generated extracellularly. It is a potent biological mediator that contributes to the protection of cells and tissues during stress conditions such as ischaemia. We had previously generated a knockout model for the A_{2a} receptor (Ledent et al. Nature 388: 674-678, 1997). Additional experiments were made in collaboration with various groups, in order to delineate further the role of adenosine receptors in various aspects of physiology.

Caffeine has biphasic effects on locomotion, and blockade of the adenosine A_{2A} receptor ($A_{2A}R$) was previously shown to be necessary for the stimulatory effect of low doses of caffeine, but not for the locomotor depressant effect observed at high doses. Ordinary caffeine consumption is not considered as a situation of drug abuse, but evidence of caffeine dependence in adult subjects has been reported. We have investigated whether the appetitive properties of caffeine can be linked to the blockade of adenosine A_{2A} receptors. Adenosine A_{2A} receptor knockout mice were compared to wild-type controls in a caffeine intake paradigm. When mice had ad libitum access to caffeine (0.3 g/l) and water in a two-bottle paradigm for 12 consecutive days, adenosine $A_{2A}R$ KO mice drank less caffeinated solution, demonstrating a reduced appetite for caffeine as compared to adenosine $A_{2A}R$ WT mice, revealing an important role for the adenosine A_{2A} receptor in the appetitive properties of caffeine (El Yacoubi et al. 2005).

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

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

Behavioral and biochemical studies have suggested a functional link between the endogenous cannabinoid and opioid systems. Different hypotheses have been proposed to explain the interactions between opioid and cannabinoid systems such as a common pathway stimulating the dopaminergic system, a facilitation of signal-transduction- and/or a cannabinoid-induced

enhancement of opioid peptide release. The functional interaction between endogenous opioid and cannabinoid receptor systems was tested in the caudate putamen and nucleus accumbens. For this purpose, we examined by autoradiography the functional activity and density of μ-, κ- and δ-opioid receptors in both brain regions of cannabinoid CB_1 receptor knockout mice. [^{35}S]GTPγS binding results showed that deletion of the CB1 cannabinoid receptor markedly increased κ-opioid (50%) and δ-opioid (42%) but not μ-opioid receptor activities in the caudate putamen. Binding autoradiography showed a similar density of μ-, κ- and δ-opioid receptors between mutant and wild-type mice. No differences were found in densities or activities of μ-, κ- and δ-opioid receptors in the nucleus accumbens. Deletion of CB1 therefore produces a pronounced increase in the activity of κ- and δ-opioid receptors in the caudate putamen. This endogenous interaction between opioid and cannabinoid receptors may be relevant to understand a variety of neuroadaptative processes involving the participation of opioid receptors, such as motor behaviour, emotional responses and drug dependence (Uriguen et al. 2005). In another study, behavioral responses induced by the stimulation of the endogenous opioid system using an inhibitor of enkephalin-degrading enzymes (RB101), were studied in CB_1 receptor knockout mice. Analgesia, locomotor activity, anxiety and antidepressant-like effects were measured after RB101 administration and similar modifications were observed in CB_1 knockout and wild-type mice. This approach did not allow therefore to support the physiological interaction between cannabinoid and opioid systems (Jardinaud et al. 2005).

Acute rewarding properties are essential for the establishment of cocaine addiction, and multiple neurochemical processes participate in this complex behavior. The self-administration paradigm was used to evaluate the role of CB_1 cannabinoid receptors in several aspects of cocaine reward, including acquisition, maintenance, and motivation to seek the drug. For this purpose, both CB_1 receptor knockout mice and wild-type littermates were trained to intravenously self-administer cocaine under different schedules. Only 25% of CB1 knockout mice vs 75% of their wild-type littermates acquired a reliable operant responding to self-administer the most effective dose of cocaine (1 mg/kg/infusion), and the number of sessions required to attain this behavior was increased in knockout mice. Animals reaching the acquisition criteria were evaluated for the motivational strength of cocaine as a reinforcer under a progressive ratio schedule. The maximal effort to obtain a cocaine infusion was significantly reduced after the genetic ablation of CB_1 receptors. A similar result was obtained after the pharmacological blockade of CB_1 receptors with SR141716A in wild-type mice. Moreover, the cocaine dose-response curve was flattened in the knockout group, suggesting that the differences observed between genotypes were related to changes in the reinforcing efficacy of the training dose of cocaine. Self-administration for water and food was not altered in CB1 knockout mice in any of the reinforcement schedules used, which emphasizes the selective impairment of drug reinforcement in these knockout mice. Finally, cocaine effects on mesolimbic dopaminergic transmission were evaluated by in vivo microdialysis in these mice. Acute cocaine administration induced a similar enhancement in the extracellular levels of dopamine in the nucleus accumbens of both CB_1 knockout and wild-type mice. This work demonstrates that CB1 receptors play an important role in the consolidation of cocaine reinforcement, although are not required for its acute effects on mesolimbic dopaminergic transmission (Soria et al. 2005).

Neuropathic pain is a clinical manifestation characterized by the presence of spontaneous pain, allodynia and hyperalgesia. The involvement of CB1 cannabinoid receptors in the development and expression of neuropathic pain was evaluated. For this purpose, partial ligation of the sciatic nerve was performed in CB_1 KO mice and their wild-type littermates, and the development of mechanical and thermal allodynia, and thermal hyperalgesia was evaluated. Pre-surgical tactile and thermal withdrawal thresholds were similar in both genotypes. In wild-type mice, sciatic nerve injury led to a neuropathic pain syndrome characterized by a marked and long-lasting reduction of the paw withdrawal thresholds to mechanical and thermal stimuli. These manifestations developed similarly in mice lacking CB_1 cannabinoid receptors. The consequences of gabapentin administration were also investigated, but similar suppression of mechanical and thermal allodynia was obtained for both genotypes. These results indicate that CB_1 cannabinoid receptors are not critically implicated in the development of neuropathic pain nor in the anti-allodynic and anti-hyperalgesic effects of gabapentin (Castane et al. 2005).

8. Characterization of GPR3

A knock out model for the orphan receptor GPR3 was established previously. Besides other aspects that are presently being studied, an important role of this receptor was identified in oocyte maturation. After becoming competent for resuming meiosis, fully developed mammalian oocytes are maintained arrested in prophase I until ovulation is triggered by the luteotropin surge. Meiotic pause has been shown to depend critically on maintenance of cAMP level in the oocyte and was recently attributed to the constitutive Gs signaling activity of GPR3. We have now shown that mice deficient for GPR3 are unexpectedly fertile but display progressive reduction in litter size despite stable age-independent alteration of meiotic pause. Detailed analysis of the phenotype confirms premature resumption of meiosis, in vivo, in about one-third of antral follicles from $GPR3^{-/-}$ females, independently of their age. In contrast, in aging mice, absence of GPR3 leads to severe reduction of fertility, which manifests by production of an increasing number of nondeveloping early embryos upon spontaneous ovulation and massive amounts of fragmented oocytes after superovulation. Severe worsening of the phenotype in older animals points to an additional role of GPR3 related to protection (or rescue) of oocytes from aging. GPR3-defective mice may therefore constitute a relevant model of premature ovarian failure due to early oocyte aging (Ledent et al. 2005).

9. Structure and function of CCR5

It became clear over the recent years that most, if not all, G protein-coupled receptors (GPCR) are able to form dimers or higher order oligomers. Chemokine receptors make no exception to this new rule and both homo- and heterodimerization were demonstrated for CC and CXC receptors. Using CCR5 and CCR2 as models, we demonstrated negative binding cooperativity between the two subunits of a dimer. The consequence is that only one chemokine can bind with high affinity onto a receptor dimer. We have published this study (El Asmar et al. 2005) as well as a review on this topic (Springael et al. 2005). We have also pursued this analysis, demonstrating that the negative cooperativity between protomers is of allosteric nature (unpublished). These observations can likely be extended to other classes of GPCRs. In collaboration with the group of Fernando Arenzana, in Paris, we have demonstrated that CCR5 displays constitutive activity, and that small molecule antagonists such as TAK-779 are inverse

agonists. Mutation of the DRY motif of CCR5 abolished its constitutive activity and agonist-induced signaling, while allowing agonist-promoted phosphorylation and beta-arrestin-dependent internalization of the receptor (Lagane et al. 2005). We have also contributed to the demonstration in a mouse model that CCR5 deficiency exacerbates T-cell-mediated hepatitis, and leads to increased levels of CCR5 ligands and a more pronounced liver mononuclear infiltrate, suggesting that CCR5 expression can modulate severity of immuno-mediated liver injury (Moreno et al. 2005).

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Role and mechanisms of synaptic “Tagging” in long-term memory.

LONG-TERM MEMORY IN MOUSE HIPPOCAMPAL SLICES

Nowadays there is a general consensus in the neuroscientists community to believe that memories are encoded in the brain thanks to changes in the strength of synaptic transmissions. A biological model of that phenomenon exists. In a thin hippocampal slice (0.5 mm thick) maintained artificially alive it is possible to induce a long-term potentiation of the strength of synapses by appropriate electrical stimulation of presynaptic fibers. This phenomenon is known as the long-term potentiation or LTP. There are two types of LTP: application of one train of high frequency stimulation (HFS) elicits a short-lasting LTP (S-LTP) which lasts 1-2 h, whereas multiple trains (3 or 4) trigger a long-lasting LTP (L-LTP) which persists longer than 4 h. The mechanisms underlying S-LTP are relatively well known. They consist of incorporation of extra chemoreceptors in the postsynaptic membrane and phosphorylation of proteins on the threonin and serin amino acids. By contrast, the mechanisms of L-LTP are poorly understood. They are the aim of the experiments currently performed in our laboratory.

1. Long-lasting LTP and metaplasticity

It was known that the induction of LTP could be modified by previous treatments applied to the neurons. This fact, called metaplasticity, means that the capability of a neuron to be involved in memory depends on its history. We recently found that the persistence of LTP (i.e. its change from a short-lasting to a long-lasting pattern) could also be influenced in a metaplastic way. We also found that the neuron's choice to use this or that particular signaling cascade also depends on the history of that neuron.

Classically, LTP is studied on hippocampal slices maintained " in interface " as well as during recovery from the trauma caused by slicing as during recordings. In the interface-slice preparation, slices are partially submerged in artificial cerebro-spinal fluid (ACSF) with the top surface of the slice exposed to a humidified atmosphere of 95% 02 and 5% CO2. In this case the slice gets oxygen that is diffused through the very thin film of liquid covering the slice. We found that if the slice was allowed to recover in submersion (in the submerged-slice preparation, slices are completely submerged in oxygenated ACSF) the properties of the LTP dramatically changed. In these conditions a single train of high frequency stimulation (100 Hz, 1 sec) triggered a long-lasting LTP instead of a short-lasting one. Everything happened as if the slice had been preconditioned to learn better. This kind of plasticity did not involve NMDA receptors but metabotropic glutamate receptors. We found that a similar phenomenon could be induced on the classical preparation (both recovery and recordings "in interface") by inhibiting SK potassium channels during the recovery period.

It was known that signaling pathways recruited in LTP could vary according to the used stimulation protocol. We found that this recruitment also depended on the history of the

neurons. Whatever recovery occurred "in interface" or "in submersion", 4 trains of stimulation triggered a long-lasting LTP. However if recovery occurred in interface the PKA signaling pathway was recruited, whereas if recovery occurred in submersion, long-lasting LTP depended on the MAPK/ERK signaling pathway.

(In course of revision in Learning and Memory).

2. Tyrosin-phosphorylation of rabphilin 3A during long-lasting LTP

Phosphorylation of serine or threonine residues is well known to play a major role in LTP. We have investigated the potential role of phosphorylation on a tyrosin residue, using a proteomic analysis. L-LTP was induced in slices by a perfusion of forskolin, an activator of synthesis of cAMP and IBMX an inhibitor of its destruction. Then we separated the proteins of samples from CA1 regions of hippocampal slices using two-dimension electrophoresis and we analysed their state of phosphorylation using an anti-phosphotyrosin antibody. Using mass spectrometry (MALDI-TOFF) after tryptic digestion for the identification of the different proteins, we found that induction of a long-lasting LTP was coupled with a tyrosin-phosphorylation of rabphilin 3A, a synaptic protein involved in exocytosis of synaptic vesicles. This phosphorylation was maximal 1 h after LTP induction. This suggests that tyrosin-phosphorylation of the presynaptic protein rabphilin 3A could play a role in the persistence of LTP.

(Manuscript submitted).

3. Overexpression of p25 protein influences L-LTP in a sex-linked way

p25, a degradation product of p35, has been reported to accumulate in the forebrain of patients with Alzheimer's disease. p25 as well as p35 are activators of cyclin-dependent kinase 5 (Cdk5) although p25/Cdk5 and p35/Cdk5 complexes have distinct properties. We have studied the influence of low-level p25 expression in hippocampal synaptic plasticity each sex separately in two different genetic backgrounds (129B6F1 and C57BL/6). Surprisingly, we found that low-level p25 expression had different consequences in male and female mutants. In the two genetic backgrounds LTP induced by a strong stimulation of the Schaffer's collaterals (four trains, 1-s duration, 5-min interval) was severely impaired in male, but not in female, p25 mutants.

(Published in European Journal of Neuroscience).

4. Synaptic tagging in L-LTP

Several pieces of evidence strongly suggest that L-LTP is critically dependent on de novo protein synthesis. This immediately poses the problem of to know how newly synthetized proteins are guided to the appropriate synapses. In 1997, Frey and Morris demonstrated that activity of synapses "tagged" them to capture the products of gene expression. During the last year, we have elaborated Frey and Morris'protocol where two distinct bundles of Schaffer

collaterals were stimulated. The main result obtained until now is that synaptic "tagging" is hindered in the aging mouse. This could be one of the major mechanisms underlying the decreased performance of memory with age.

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Progress Report of the Research Group of

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Roles of the direct and indirect pathways in functions and disorders of the basal ganglia.

I. Direct and Indirect pathways in basal ganglia functions and disorders

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

Our aim was to generate transgenic mice allowing the study of the specific roles of striatopallidal or striatonigral neurons. We have obtained mice strains expressing the Cre recombinase under the control of the A_{2A} receptor promotor inserted in a BAC (bacterial artificial chromosome) to direct gene expression specifically in striatopallidal neurons. These lines have been crossed with a reporter strain (Rosa26-LacZ) in order to determine whether they selectively expressed Cre in these striatopallidal neurons. Co-localisation experiments using anti-enkephalin antibody (Enk) and LacZ activity detection showed that >80% of the LacZ neurons are also Enk+. Retrograde labelling of striatonigral neurons combined with the immunodetection of β-galactosidase showed that striatonigral are LacZ-negative. The same lines of CRE mice have been also crossed with three different lines of reporter mice allowing the expression of GFP or analogues proteins. One of these demonstrated the expression of GFP restricted in a subpopulation of striatal neurons which were never identified as striatonigral by retrograde labelling and who co-expressed D2 and A_{2A} receptors as demonstrated by single cell RT-PCR. Therefore, altogether, these results demonstrated that we have generated strains of transgenic mice with a specific Cre expression in the striatopallidal neurons. The striatopallidal-GFP mice will now both allow to specifically isolate this population of striatal neurons by using dissociation and sorting and to specifically record them by using patch clamp.

Having these neuron-specific transgenic lines, we have crossed them with strains of "floxed" mice allowing the selective inactivation of genes in this population of neurons. The NMDA receptors seem to play a key role in reward and drug addiction (ventral striatum) and in motor learning (dorsal striatum). Moreover, NMDA receptors are supposed to be involved in synaptic plasticity at the cortico-striatal and cortico-accumbal synapses (LTP and LTD). Synaptic plasticity in these areas is strongly suggested to be the basis for motor learning and addiction. An important question is therefore the specific role of this receptor in each subpopulation. The NR1 floxed mice has been previously generated and published and have been obtained from Prof. Tonegawa and collaborators (MIT, Boston, USA) allowing a conditional inactivation of NR1 by the Cre recombinase. These mice have been double-crossed with our A2A-Cre mice to obtain homozygous mice. These resulting mice are tested for the specific inactivation of NR1 in striatopallidal neurons by using in situ hybridization, immunohistochemistry and binding. Two other genes of interest for their roles in the basal ganglia physiology have been chosen and corresponding floxed genes mice have been obtained. Their breeding and crosses with our Cre mice have been started.

Regulation of striatal neurons excitability and of corticostriatal transmission and plasticity

We previously studied the modulation of the glutamatergic corticostriatal transmission and plasticity by A_{2A} receptor. We have now pursuied this study by the analysis of the mechanisms

of the modulation exerted by D2 and A_{2A} receptors on the striatal neurons excitability and corticostriatal transmission and plasticity in normal and hypodopaminergic conditions. We showed that D2 receptor activation decreased striatal neurons excitability, an effect reversed by co-stimulation of A_{2A} receptor, through a pathway probably involving subtype of calcium channels (CaV1.3). We also showed that on striatal neurons, the voltage gated sodium channels could be modulated through the phospholipase C transduction pathway and that proteine kinase C and IP3 receptor activations regulate its activity in opposite ways (6).

Neuronal death and neuroprotection in models of Huntington's disease

We previously developed an experimental model of Huntington's disease in rat or mouse by using subchronic injection of 3-nitropropionic acid (3NP). We previously demonstrated the high reproducibility of this model in terms of lesion and evaluated the potential neuropotective effects of adenosine A_{2A} or A_1 receptors ligands. Minocycline has been also proposed as a neuroprotective agent in several models of neurodegenerative diseases. We evaluated its neurprotective potency in different models of Huntington's disease and demonstrated that it could slow down the development of inflammation and of caspases-induced neuronal death but not the development of calpain-dependent neuronal (1).

Identification of a new brain anti-opioid system

We have take part to the identification of prolactin-releasing peptide and its receptor GPR10 as a new neuropeptidergic system exhibiting anti-opioid effects (7).

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

We had characterized cellular mechanisms leading to the alterations observed in calretinin-deficient mice through an approach combining electrophysiology in vitro (patch clamp in the perforated patch configuration) of granular cells of the cerebellum and computer modeling. We demonstrated that the absence of calcium buffering modifies the intrinsic excitability through a modification of the response of calcium-activated potassium channels. We extended these data by using different mathematical models and reviewed these results in a general perspective (4). Further, we asked whether an increase in neuronal calcium buffering capacity would have consequences on neuronal excitability.. Using these mathematical models, we suggested that this increase dramatically changes the firing pattern of modeled granule cell from a regular spiking to different types of bursting firing (8). This theoretical suggestion has been fully verified experimentally by loading different concentrations of the exogenous calcium buffer BAPTA through the patch clamp pipette in granule cells recorded in brain slices (8). This suggests that subtle and local modifications in calcium buffering capacity could dramatically change the mode of neuronal coding .

Members of the laboratory also take part to a work describing the molecular mechanisms of long term potentiation induction at the mossy fiber-granule cell synapse (5).

The absence of calretinin in cerebellar granule cells constitute a main hypothesis consistent with the perturbations that we previously demonstrated in $Cr^{-/-}$ mice. To investigate this

hypothesis, we specifically rescued the expression of calretinin in the cerebellar granule cells of $Cr^{-/-}$ mice. The calretinin expression was targeted to cerebellar granule cells by using a fragment of the gene coding for the $GABA_A\alpha$ 6 subunit encompassing the promoter and the exons 1 to 8. This part of the gene has been previously shown to allow restricted transgene expression in cerebellar granule cells. We obtained several lines of transgenic $Cr^{-/-}$ mice exhibiting a selective and restricted re-expression of calretinin in granule cells as demonstrated by in situ hybridization, RT-PCR and imunohistochemistry (2). In vitro experiments using patch clamp technique in these strains of mice demonstrated that the rescue of calretinin expression in granular cells restores a normal intrinsic excitability of these neurons. Moreover, in vivo electrophysiology experiments demonstrated that the rescue of calretinin in granule cells restores a normal firing behavior of Purkinje cells recorded in alert mice. Finally, behavioural analysis of the motor coordination also showed that the rescue expression of calretinin only in cerebellar granule cells is sufficient to restore a normal phenotype for all parameters (2).

We described a fast (160 Hz) local field potential oscillation recorded in vivo through extracellular recordings in the cerebellar cortex of mice deficient in calcium binding proteins (Cheron et al., 2004, 10). We suggested that this oscillation was generated by Purkinje cells whose behavior became rythmic and synchronous in these mice. We have pharmacologically identified NMDA and GABAa receptors as well as gap junctions as requested to generate this oscillation. This constitutes the first description of a fast oscillation in the cerebellum whereas such electrophysiological behaviors have been reported in other brain areas such as cerebral cortex, hippocampus and thalamus where it is proposed that they play important functional roles (Cheron et al., 2004). We have also showed that in mice deficient in both calbindin and parvalbumin, a calcium buffer exhibiting a slower kinetic and a distribution including interneurons and Purkinje cells, a double oscillation is detected. Each phase of this double oscillation is phase-locked to the frequency and the rythmicity of one population of Purkinje cells (10).

In all these studies on cerebellar physiology, we noted a rather high difficulty to obtain reproducible results in terms of locomotor behavior. We therefore analysed it carefully in mice from different ages and genetic backgrounds (3) as well as in mice chronically treated with ethanol (9). We detected significant differences that pointed out the need of carefulness in the choice of the adequate mouse models.

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EVALUATION OF LEPTIN ANTAGONISTS FOR TREATMENT OF MULTIPLE SCLEROSIS

1. ANALYSIS OF LEPTIN RECEPTOR COMPLEX FORMATION AND ACTIVATION

The 16 kDa. cytokine-like hormone leptin is mainly produced and secreted by adipocytes. Protein levels positively correlate with the body fat energy stores. Spontaneous mutations that lead to a functional defect in the hormone production lead to a morbid obese phenotype caused by a marked increase in food intake and a strongly reduced energy expenditure. Besides this body-weight regulation, leptin also plays an important role in the control of immune responses, and possibly in the onset of several auto-immune diseases. Examples include multiple sclerosis, rheumatoid arthritis, type I diabetes, and Crohn's disease (Peelman et al. 2004, Prog Lipid Res 43, 283-301; ibid., 2005).

Leptin mediates its effects upon binding and activation of the single-subunit leptin receptor (LR). The LR long form, the only isoform capable of signaling, is highly expressed in hypothalamic neurons, in line with its role in regulating metabolism. In addition, expression at functional levels could also be shown in several other cell types including hepatocytes, lung, testis, immune cells, etc, thereby forming the basis for the peripheral biological functions of leptin. Like all members of the class I cytokine receptor family, the LR lacks intrinsic kinase activity and uses cytoplasmic associated Janus kinases (JAKs). Several signaling cascades can be activated, including the STAT3 (signal transducer and activator of transcription 3) pathway. Extracellularly, the LR consists of two so-called cytokine receptor homology (CRH) domains, CRH1 and CRH2. Both are separated by an immunoglobulin-like (Ig-like) domain, and are followed by two additional fibronectin type III (FN-III) modules close to the membrane. CRH2 is the major leptin binding domain. The FN-III and Ig-like domains do not show detectable affinity for the ligand, but are essential for the formation of an activated receptor complex (Fong et al. 1998, Mol Pharmacol 53:234-40; Zabeau et al. 2004, Mol Endocrinol 18, 150-61).

It was long believed that the LR, like most cytokine receptors, becomes activated upon simple ligand-induced homodimerization. Several data sets strongly contradict this model. First, the receptor appears as pre-formed oligomers on the cellular surface (e.g. Couturier and Jockers 2003, J Biol Chem 278, 26604-11). This clustering appears to be disulfide-linked, and most likely involves cysteine residues in the membrane-proximal CRH2 domain (Zabeau et al., 2005). Second, a detailed mutagenesis study revealed three binding sites on leptin, sites I-III. Mutations in binding site I show a modest effect on signaling and do not affect binding to CRH2. Mutations of binding site II impair binding but have only limited effect on signaling. Site III residues appear not to be involved in ligand binding to CRH2, but are essential for receptor activation (Peelman et al. 2004, J. Biol Chem 279, 41038-46). This configuration very closely mimics the hexameric interleukin 6 (IL6) receptor complex. Third, we used a signaling complementation strategy to demonstrate that the LR becomes activated upon higher order clustering, i.e. more than two receptors per activated complex. In this experimental set-up, two signaling deficient LR mutants, one unable to activate the JAKs, the other impaired in STAT3 recruitment, are only able to signal when they are co-expressed. No complementation occurs when the extracellular domains of both mutants is replaced by that of the strictly

homodimeric erythropoietin receptor. Taken together and given the intrinsic needs for JAK-STAT signaling (i.e. two JAK activating receptors, and at least one STAT recruiting receptor) these data can only be explained by assuming a higher order clustering of the LR (Zabeau et al. 2004, Mol Endocrinol 18, 150-61). Finally, we recently could demonstrate an important role for the FN-III domains in receptor activation. A deletion variant with an extracellular domain consisting of only these domains is constitutively active. When the FNIII domains are expressed, they form disulfide-linked oligomeric complexes in solution. Mutation of the two conserved cysteines in the FN-III domains results in complete impairment of JAK activation and hence signaling, without any affect on ligand binding (Zabeau et al., 2005).

We used all these data-sets to propose a model for LR activation (Figure 1). Herein, the receptor exists as preformed dimers, based on homotypic, likely disulfide-linked, interactions between the CRH2 domains of two LRs. In this configuration, the FN-III domains, and therefore also the cytoplasmic receptor tails and thus the JAKs, are held apart spatially, preventing constitutive, ligand-independent JAK activation. Based on the previous observation that the receptors become activated upon higher order clustering, we propose that two leptin molecules cluster two such LR dimers.

We now also mapped the three interaction sites of leptin on the LR in great detail (Iserentant et al., 2005; Peelman et al., submitted). The combined data of inter-molecular LR disulfide bridges and of the position of these three binding sites in the LR lend further support for the concept that leptin forms a 2:4 hexameric complex with its receptor. A molecular model was built for the leptin/receptor complex. In this model, leptin first binds to a disulfide-linked dimeric LR. Binding site I of leptin interacts with the CRH2 domain of the first LR chain, binding site II interacts with the CRH2 domain of the second LR chain. Activation of the receptor then requires dimerisation of these 1:2 leptin:LR trimers. This hexamerisation involves interactions of binding site III of the leptin molecule of a first trimer with the LR Ig-like domain of a second trimer (Peelman et al., submitted). Ligand binding thus brings the FN-III domains of two different preformed dimers in close proximity, whereby subsequent S-S bridge formation may fix the complex in the correct orientation, leading to signal transduction.

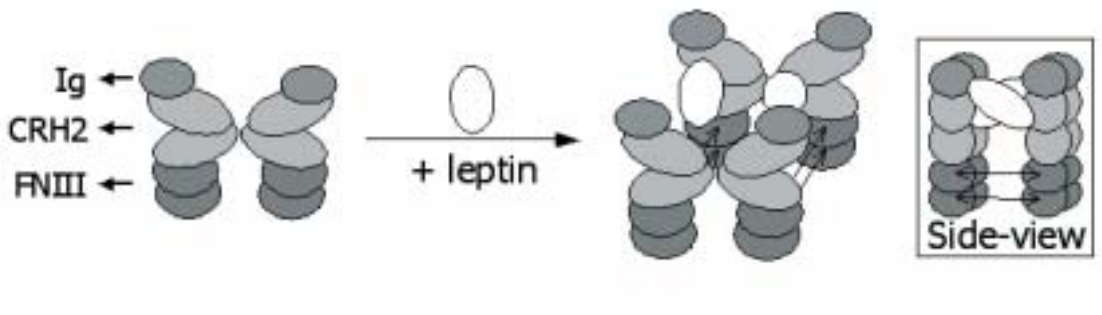

Figure 1: Model for LR clustering and activation

2. OPTIMISATION OF LEPTIN MUTEINS WITH ANTAGONISTIC PROPERTIES

Mutations in binding site III of leptin interfere with the hexamerisation process and thus with receptor activation. A S120A/T121A binding site III leptin mutant still binds to the receptor, but is unable to activate the receptor (Peelman et al. 2004, J. Biol Chem 279, 41038-46). It therefore acts as a competitive inhibitor of leptin receptor signaling.

This mutein is efficiently expressed in E. coli in inclusion bodies using the Pet-11a system, and a purification protocol including a refolding step is in place (figure 2). To enhance the *in vivo* serum half-life, we now also plan to PEGylate this leptin mutein. In this respect, several cysteine mutants of this leptin antagonist have been developed: this includes adding one extra cysteine at the N-terminus or at the C-terminus. In addition, we also replaced the S120 or T121 residue by a cysteine, anticipating that adding a bulky PEG group at these positions will, as is the case for the antagonist, interfere with the LR site III interaction.

All these mutants can also be expressed in E. coli at expression levels > 50 mg/liter.

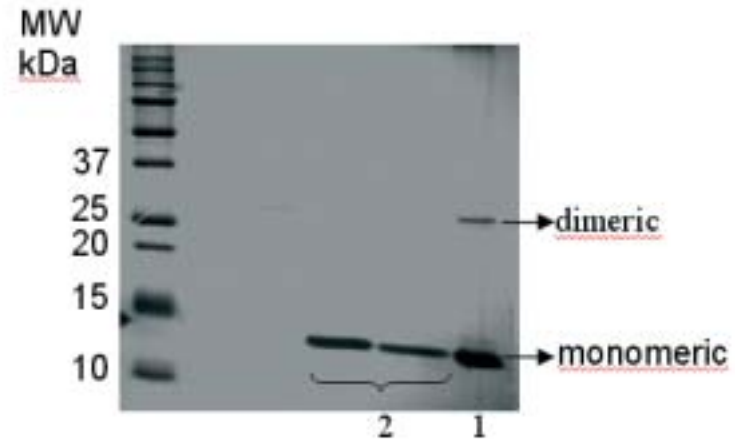

Figure 2: SDS-PAGE with silverstaining of refolded S120A/T121A mouse leptin mutein.
1: Sample after refolding of protein isolated from inclusion bodies. 2: Sample after gelfiltration.

3. DEVELOPMENT OF ANTAGONISTIC NANOBODIES TARGETING THE LEPTIN RECEPTOR

The observation that additional LR-LR interactions are involved in the formation of an activated hexameric leptin/LR complex opens the possibility to block leptin signaling without interfering with ligand binding. A major concern in the development of leptin-based therapeutic strategies for autoimmune diseases is that complete LR blockage also interferes with leptins' hypothalamic body weight regulating role. Indeed treatment of mice with the S120A/T121A leptin mutein induces weight gain (Peelman et al. 2004, J. Biol Chem 279, 41038-46). It is likely that the blood-brain-barrier (BBB) physically separates the peripheral (i.e. on immune cells) and central (i.e. on hypothalamic neurons) leptin activities. An active leptin BBB transport model has recently been suggested, but the transporter system itself has not yet been identified (Banks 2002, Brain Res 950, 130-6). Possibly, a short isoform of the LR that lacks most of the cytosolic domain plays a modulating role by trapping and transferring the ligand to the actual transporter system. We aim to develop a leptin antagonist that does not interfere

with leptin transport, and that cannot cross the BBB itself. Such antagonist may selectively inhibit the peripheral functions of leptin. Based on our hexameric leptin/LR model, we postulate that molecules directed against the FN-III or Ig-like domains could fulfill such role.

We chose to use the "nanobody" technology for the development of such LR antagonists. Nanobodies are the variable domain of a class of antibodies found in camels and llamas that are only composed of heavy chains. Nanobodies have relatively long complementarity determining regions (CDRs) that allow binding to regions that lie deeper inside protein (complexes) (Desmyter et al. 1996, Nat Struct Biol 3, 803-11). Llamas were immunized with affinity purified extracellular part of the murine LR in collaboration with Ablynx. At certain time points, blood was collected and lymphocytes were isolated. cDNA was prepared and used to amplify the sequences coding for the nanobodies. Fragments were cloned in de Pax51 expression vector, which allows bacteriophage surface expression of the nanobodies as geneIII (a phage coat protein) fusion-proteins. Thus far, LR-specific phages were enriched in four different screens (thereby varying washing and panning conditions, e.g. on LR(subdomain)-expressing cells or on plates with immobilized purified LR protein). In each screen, phages of the second and third panning round were used to re-infect TG1 cells. Nanobody fusion-proteins were isolated from selected clones by preparing periplasmatic extracts.

These extracts were initially tested for neutralizing leptin activity. HEK293T cells were transfected with the LR and the STAT3-responsive rPAP1-luciferase reporter construct. Transfected cells were stimulated with suboptimal concentrations of leptin, in the presence or absence of a serial dilution of nanobody extracts. Some nanobodies gave a clear reduction in leptin signaling, while others had no effect (an example in shown in figure 3, left). As a control, the effect on leukemia inhibitory factor (LIF), a related class I cytokine that also signals via STAT3 but via a different receptor complex, was tested. None of the nanobodies inhibited LIF signaling (panel B). 21 such neutralizing nanobody clones were identified and sequenced.

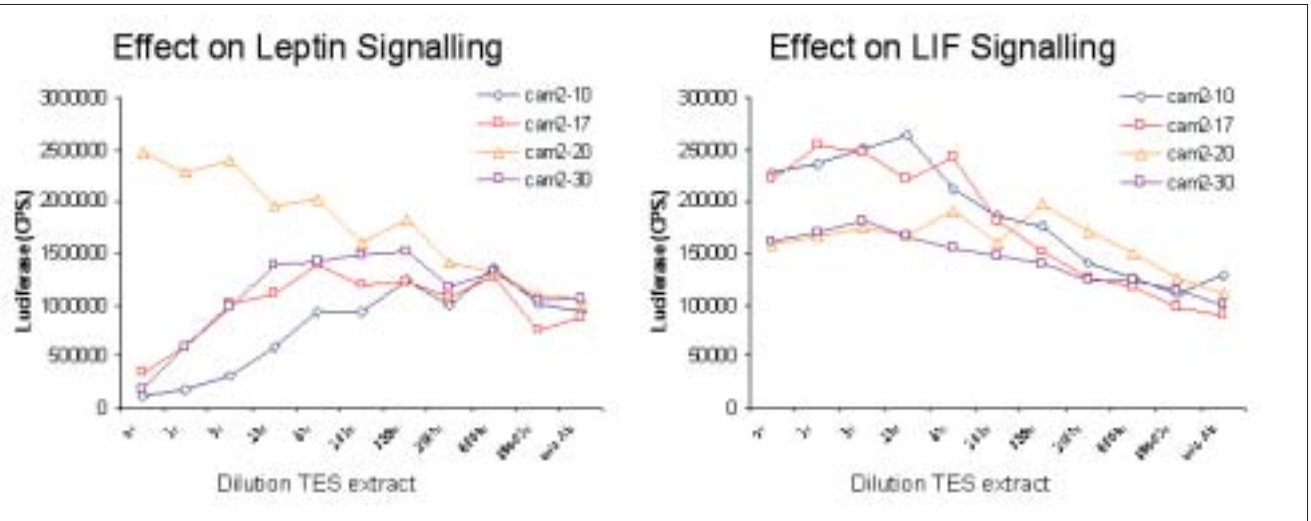

Figure 3: Effect of nanobodies on leptin (left) and LIF (right) signaling

Selected nanobodies were then tested for their ability to block leptin binding. Cos1 cells transfected with the LR were incubated with a leptin-SEAP (secreted alkaline phosphatase) fusion-protein, with or without nanobody extract. Cells were washed and bound enzymatic activity was measured and counted as measure for leptin binding. Three out of the 21 nanobodies gave a clear effect on leptin binding, thus providing an explanation for their neutralizing effect. Data for one such clone (cam 2.17) is shown in figure 4.

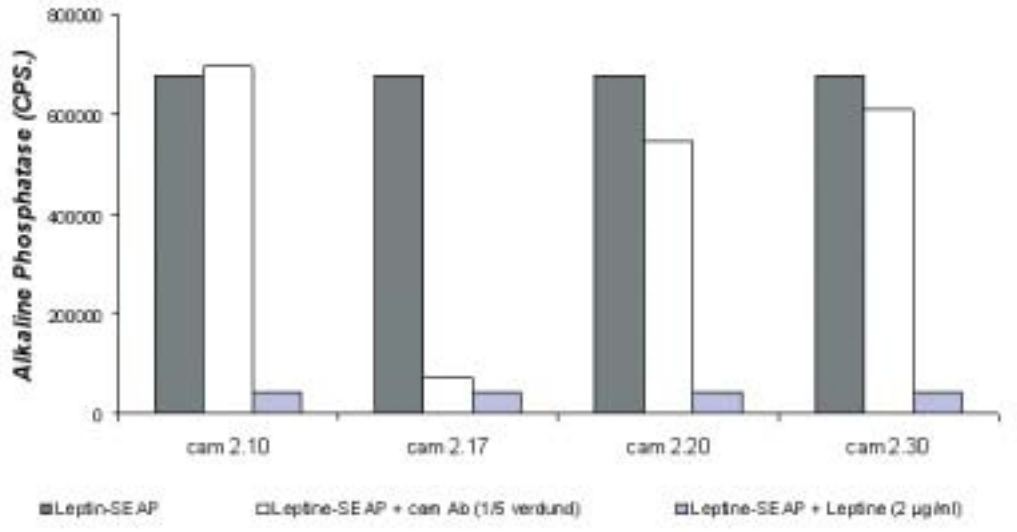

Figure 4: Effect of nanobodies on leptin binding

We next determined which LR sub-domain was bound by the selected nanobodies. The different LR sub-domains were fused to SEAP, and expressed as soluble proteins. Nanobodies were immobilized on plates and incubated with supernatants containing the different SEAP fusion-proteins. Bound alkaline phosphatase activity was measured after washing the plates. Results indicated that 13 nanobodies were directed against the Ig-like domain, 3 against CRH2, and 5 against FN-III (Figure 5). As expected, the three nanobodies that blocked leptin binding were directed against CRH2, being the major leptin binding site.

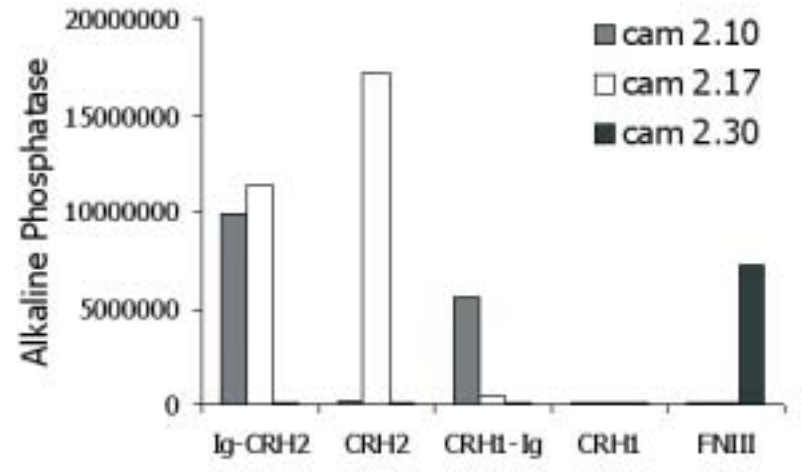

Figure 5: Domain mapping

In a similar way, we also determined the nanobody affinities for the receptor. Plate bound nanobodies were incubated with a serial dilution of affinity purified LR extracellular domain coupled to SEAP. Affinities were calculated with the GraphPad software. For most nanobodies affinities are in the lower nM range (0,3 - 10 nM).

In conclusion, we obtained 21 nanobodies that interfere with leptin but not LIF signaling. The majority binds to the Ig-like domain, while 5 are directed against the FN-III domains. The 3 nanobodies that bind to CRH2 block leptin binding to the receptor. It thus appears to be possible to inhibit leptin signaling, without interfering with ligand binding, thus confirming our model for LR activation.

The most relevant nanobodies were subcloned as mouse albumin fusion proteins (DNA: universal signalpeptide - HA tag - nanobody - flexible GGS linker - albumin) in the pMET7 expression vector, which allows high expression in eukaryotic cell systems. Such fusion proteins show enhanced serum half-life properties *in vivo*. Furthermore, the albumin extravasation effect leads to enhanced accumulation at sites of inflammation. Fusion-proteins were transiently produced in Cos1 cells and supernatant was tested for inhibition of leptin signaling on HEK293T cells transfected with the LR (see above). Based on these data, the strongest neutralizing nanobodies directed against the Ig-like, CRH2, and FN-III domains were selected. The neutralising effect of one of the purified anti-Ig-like nanobodies (the cam 4.10-albumin fusion protein) is shown in figure 6. A clear dose-dependent inhibition of leptin signaling was obtained.

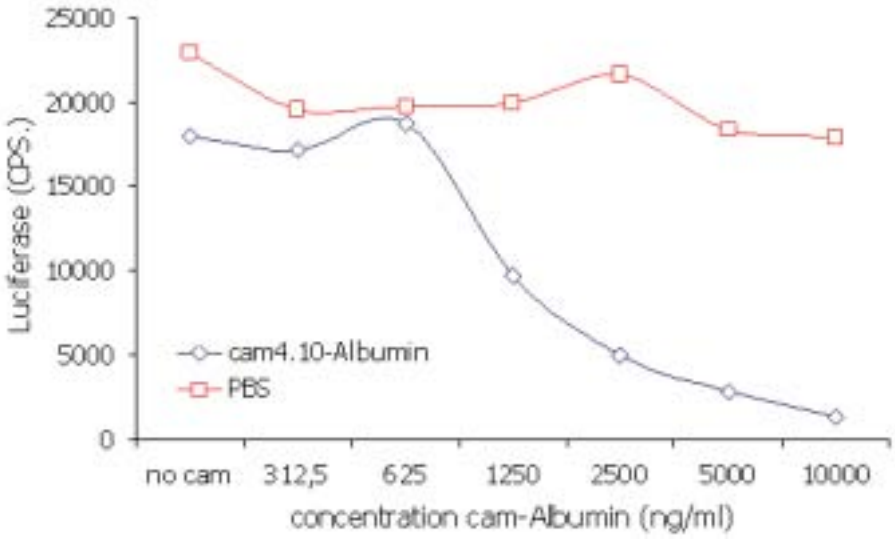

Figure 6: Neutralization by cam4.10-Albumin

4. FUTURE PROSPECTS

In a next step, the different antagonists will be evaluated *in vivo*. First, the effect on body weight regulation and immune function in normal mice will be monitored. This should be indicative for their mode of action, e.g. selective peripheral vs. central activity. Based on these observations, large-scale production of selected antagonists will be initiated. Later on, these proteins will be analyzed in model systems for multiple sclerosis.

Since we have obtained nanobodies directed against every relevant sub-domain of the LR, these can also be useful tools to study the transport of leptin across the BBB. A relatively straightforward transcytosis assay in MDCK will first be used to set up the conditions for leptin transcytosis. MDCK cells were therefore already stably transfected with the short isoform of the LR. At a later stage, we will extend these studies to a physiologically more relevant assay for leptin BBB transport using rat brain micro-capillaries.

5. PUBLICATIONS DIRECTLY RELATED TO THE PROJECT

1. PEELMAN, F.*, ISERENTANT, H.*, EYCKERMAN, S., ZABEAU, L. & TAVERNIER, J.
   Leptin, immune responses and autoimmune disease: perspectives on the use of leptin antagonists.
   * Shared co-authorship
   Curr. Pharm. Des. 11, 539-548 (2005) (IF=5.6).
2. ISERENTANT, H.*, PEELMAN, F.*, DEFEAU, D., VANDEKERCKHOVE, J.,
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   * shared co-authorship
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3. ZABEAU, L., DEFEAU, D., ISERENTANT, H., VANDEKERCKHOVE, J., PEELMAN, F. & TAVERNIER, J.
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   J. Biol. Chem, 280, 22632-22640 (2005) (IF=6.5).
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   Curr Med Chem 13, 199-222 (2006) (IF=4.4).
5. PEELMAN, F. COUTURIER, C., DAM, J. ZABEAU, L., TAVERNIER, J. &
   JOCKERS, R.
   New pharmacological perspectives for the leptin receptor.
   Trends Pharmacol Sci, in press (IF=13.1).
6. PEELMAN, F.*, ISERENTANT, H.*, DESMET, A.-S., VANDEKERCKHOVE, J., ZABEAU, L. & TAVERNIER, J.
   * shared co-authorship
   Mapping of Binding Site III in the leptin receptor and modeling of a hexameric leptin: receptor complex.
   J. Biol. Chem., submitted in revised version (IF=6.5).

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Molecular genetics and biology of Charcot-Marie-Tooth neuropathies

We aimed to find chromosomal loci and genes in which mutations result in known or unknown forms of inherited peripheral neuropathies. In 2005 we reported the identification of 2 novel genes: *DNM2* for dominant intermediate CMT (DI-CMTB) and *SEPT9* for hereditary neuralgic amyotrophy (HNA). Mutation screenings and genotype/phenotype correlations were performed in a large cohort of patients (mutations are updated on http://www.molgen.ua.ac.be/CMTMutations). We performed differential gene expression profiling by comparing the transcriptome of sensory and motor neurons. We provided cDNA libraries that were helpful in characterising the expression of the genes we identified.

Identification of two novel genes for inherited peripheral neuropathies:

Intermediate Charcot-Marie-Tooth (CMT) neuropathy is a genetic and phenotypic variant of classical CMT, characterized by intermediate nerve conduction velocities and histological evidence of both axonal and demyelinating features. Intermediate CMT is a genetically heterogeneous entity. Patients with mutations in several known CMT genes (e.g. *MPZ, NF-L, GDAP1, GJB1)* could present with an intermediate CMT phenotype at the electrophysiological and neuropathological level. However, there are clinically well-documented dominant intermediate CMT (DI-CMT) families mapped to new genetic regions (DI-CMTA, B and C). We refined the locus associated with DI-CMTB on chromosome 19p12-13.2 to 4.2Mb in three unrelated CMT families originating from Australia, Belgium and North America. After screening candidate genes, we identified unique mutations in the gene coding for dynamin 2 (*DNM2*) in all families. *DNM2* belongs to the family of large GTPases and is part of the cellular fusion-fission apparatus. In transiently transfected cell lines, mutations of DNM2 substantially diminish binding of DNM2 to membranes by altering the conformation of the beta3/beta4 loop of the pleckstrin homology domain. Additionally, in the Australian and Belgian pedigrees, which carry two different mutations affecting the same amino acid, Lys558, CMT co-segregated with neutropenia, which has not previously been associated with CMT neuropathies (1). This project was performed in close collaboration with S. Züchner (Duke University, Durham, USA)

Hereditary neuralgic amyotrophy (HNA) is an autosomal dominant recurrent focal neuropathy. We previously assigned the major locus for HNA to a 1.8Mb linkage interval on chromosome 17q25. We further delineated the critical region by genetic fine mapping of informative recombinations and allele sharing methods resulting in a refinement of the locus to a ~600kb candidate region. This region contains only two known candidate genes: SEC14-like 1 (S. *cerevisiae SEC14L1)* and septin 9 (*SEPT9*). We detected two missense mutations in the *SEPT9* gene, R88W in four HNA families and S93F in one HNA family. In addition, in one pedigree we found a mutation in the 5'untranslated region of *SEPT9* (c.-131C>G). All mutations co-segregated with the HNA phenotype. None of the mutations was found in 500 control individuals. Both missense mutations are located in the same stretch of 15 consecutive highly conserved amino acids. Septins are involved in cytokinesis, tumorigenesis and a number of other cellular processes. The C-terminus of the SEPT9 protein shows typical features of the septin protein family while the N-terminus, containing the HNA-associated mutations, does not

show a high degree of homology to any other known protein, making inferences about the function difficult (2).

Genotype-phenotype correlations:

Inherited peripheral neuropathies are common monogenetically inherited diseases of the peripheral nervous system. In the most common variant, i.e., the hereditary motor and sensory neuropathies (HMSN), both motor and sensory nerves are affected. In contrast, sensory abnormalities predominate or are exclusively present in hereditary sensory and autonomic neuropathies (HSAN). HSAN are clinically and genetically heterogeneous and are subdivided according to mode of inheritance, age of onset and clinical evolution. In recent years, 6 disease-causing genes have been identified for autosomal dominant and recessive HSAN (*SPTLC1, RAB7, HSN2, NTRK1, IKBKAP, NGFB*). Vesicular transport and axonal trafficking seem important common pathways leading to degeneration of sensory and autonomic neurons. We reviewed the HSAN related genes and their biological role in the disease mechanisms leading to HSAN (3).

Congenital insensitivity to pain with anhydrosis or hereditary sensory and autonomic neuropathy type IV (HSAN IV) is the first human genetic disorder implicated in the neurotrophin signal transduction pathway. HSAN IV is characterized by absence of reaction to noxious stimuli, recurrent episodes of fever, anhydrosis, self-mutilating behaviour and often mental retardation. Mutations in the neurotrophic tyrosine kinase, receptor, type 1 (*NTRK1*) are associated with this disorder. We reported 4 homozygous mutations, 2 frameshift (p.Gln626fsX6 and p.Gly181fsX58), 1 missense (p.Arg761Trp) and 1 splice site (c.359+5G>T) mutation in 3 HSAN IV patients. The splice site mutation caused skipping of exon 2 and 3 in patient's mRNA resulting in an in-frame deletion of the second leucine-rich motif. *NTRK1* mutations are only rarely reported in the European population (4).

Small heat shock proteins (small HSPs) are molecular chaperones that protect cells against stress by assisting in the correct folding of denatured proteins and thus prevent aggregation of misfolded proteins. Small HSPs also modulate apoptotic pathways by interacting with components of programmed cell death. Furthermore, some small HSPs interact with the cytoskeleton to assist in spatial organization and dynamics of its structural elements. The role of small HSPs has been studied in many disorders, including neurodegenerative disease. We recently demonstrated that mutations in *HSPB1* (*HSP27*) and *HSPB8 (HSP22),* two members of the small HSP superfamily, are associated with inherited peripheral neuropathies. We reviewed the current knowledge of small HSPs, in particular HSPB1 and HSPB8, and discussed their role in health and disease (5).

Differentially Expressed Genes in Motor and Sensory Neurons:

We performed differential gene expression profiling in the peripheral nervous system by comparing the transcriptome of sensory neurons with the transcriptome of lower motor neurons. Using suppression subtractive cDNA hybridization, we identified 5 anonymous

transcripts with a predominant expression in sensory neurons. We determined the gene structures and predicted the functional protein domains. The 4930579P15Rik gene encodes for a novel inhibitor of protein phosphatase-1 and 9030217H17Rik was found to be the mouse gene synaptopodin. We performed *in situ* hybridization for all genes in mouse embryos, and found expression predominantly in the primary class of sensory neurons. Expression of 4930579P15Rik and synaptopodin was restricted to craniospinal sensory ganglia. Neither synaptopodin, nor any known family member of 4930579P15Rik, has ever been described in sensory neurons. The identification of protein domains and expression patterns allows further functional analysis of these novel genes in relation to the development and biology of sensory neurons (6).

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Research Articles acknowledge GSKE

Research Activities 2005:

Scientific Prizes:

- Joy Irobi: Laureaat van de Tweejaarlijkse Prijzen van de Onderzoeksraad Universiteit Antwerpen, De Prijs Vandendriessche, 7 december 2005
- Kristl Claeys: Prijs van de beste presentatie tijdens de "International Symposium on Neuromuscular diseases", Brussels, October 14-15, 2005

Master thesis:

- Steven Broeckx: 'Moleculair genetische analyse van de ziekte van Charcot-Marie-Tooth type 2G', licentiaat Biochemie 2004-2005
- Ann Crosiers: 'Mutatieanalyse van het EGR2- en DNM-2 gen in Charcot-Marie-Tooth neuropathie', licentiaat Biochemie 2004-2005

Invited lectures at international meetings:

- Peter De Jonghe: Focused Workshop "Advance in the understanding of genetic neuropathies: Chacot-Marie-Tooth disease - clinical and genetic update": Recent genetic advances in CMT. 9th Congress of the European Federation of Neurological Societies (EFNS), Athens, Greece, September 17-20, 2005: invited speaker
- Peter De Jonghe: "Diagnostic guidelines for mutation screening in inherited peripheral neuropathies". Kongress: Diagnose und Management von Gangstörungen als interdisziplinäre Herausforderung, Graz, Austria, September 30-October 2, 2005: invited speaker
- Peter De Jonghe: "Charcot-Marie-Tooth and Hereditary Motor Neuropathies". International Symposium on Neuromuscular Diseases, Joint meeting of Belgian Neurological Society and Belgian Society for Pediatric Neurology, Belgian Society for Clinical Neurophysiology and Belgian Neuropathology Group, October 14-15, 2005: invited speaker
- Eva Nelis: EQA scheme organisers and assessors meeting, Manchester, UK, December 5-6, 2005: invited participant.
- Vincent Timmerman: "Congrès CMT-France. Invité d'Honneur: Progrès Récent dans la génétique moléculaire des maladies de Charcot-Marie-Tooth". Lille, France, March 19, 2005: invited speaker
- Vincent Timmerman: "Molecular genetics and biology of inherited peripheral neuropathies". Kongress: Diagnose und Management von Gangstörungen als interdisziplinäre Herausforderung, Graz, Austria, September 30-October 2, 2005: invited speaker
- Vincent Timmerman: "Molecular genetics and biology of inherited peripheral neuropathies". Annual Meeting of the Study Group Neurochemistry: Neural Signal Transduction in Health and Disease - Cytokines, mitochondrial dysfunction and transport processes. Leipzig, Germany, October 6-8, 2005: invited speaker

Presentations at international meetings:

- Kristl Claeys: "Mutations in mitofusin 2 are a major cause for autosomal dominant axonal Charcot-Marie-Tooth neuropathy". Meeting of the Peripheral Nerve Society, Tuscany, Italy, July 9-13, 2005: oral presentation
- Kristl Claeys: "Mutations in mitofusin 2 are a major cause for autosomal dominant axonal Charcot-Marie-Tooth neuropathy". International Symposium on Neuromuscular diseases in Brussels, October 14-15, 2005: oral presentation
- Ines Dierick: "Small heat shock proteins in motor and sensory neuron disease". 2nd International Congress on Stress Responses in Biology, Tomar, Portugal, September 24-28, 2005: oral presentation

- Ines Dierick: "*In vitro* aggregation of stress proteins is associated with inherited motor neuropathies". FENS/Hertie Winter School: "Neurodegenerative aggregation Disorders: From genes to protein aggregation, Neuronal dysfunction and Experimental Treatments. Kitzbühel, Austria, December 11-18, 2005: poster presentation
- Ines Dierick: "Small heat shock proteins in motor and sensory neuron disease". Departmental Research Symposium, University of Antwerp (UA), Department of Biomedical Sciences, Antwerpen, Belgium, May 24, 2005: poster presentation
- Eva Nelis: Mutation Detection meeting (HUGO), Santorini, Greece, May 31 -June 4, 2005: participant.
- Joy Irobi: "*In vitro* expression of small heat shock protein HSPB8 and HSPB1 mutations causing axonal neuropathy". 6th BiAnnual Meeting of the Belgian Society for Neuroscience, Brussels, Belgium, May 20, 2005: oral presentation
- Joy Irobi: "In vitro analysis of small heat shock protein HSP22 and HSP27 mutations causing axonal neuropathy". International Congress on Stress Responses in Biology, Tomar, Portugal, September 24-28, 2005: oral presentation
- Joy Irobi: "Functional characterization of small heat shock protein HSPB8 and HSPB1 mutations causing axonal neuropathy. 35th Annual Meeting of the Society for Neuroscience 2005, Washington DC, USA, November 12-16, 2005": oral presentation
- Joy Irobi: "*In vitro* expression of small heat shock proteins HSPB8 and HSPB1 mutations causing axonal neuropathy". FENS/Hertie Winter School: "Neurodegenerative aggregation Disorders: From genes to protein aggregation, Neuronal dysfunction and Experimental Treatments. Kitzbühel, Austria, December 11-18, 2005: poster presentation
- Vincent Timmerman: "Mutations in Mitofusin 2 are a major cause for autosomal dominant axonal Charcot Marie tooth Neuropathy". 2005 North American CMT Consortium meeting, London Ontario, Canada, May 19-21, 2005: oral presentation
- Nathalie Verpoorten: "Synaptopodin and 4 novel genes identified in primary sensory neurons". Departmental Research Symposium, University of Antwerp (UA), Department of Biomedical Sciences, Antwerpen, Belgium, May 24, 2005: oral presentation

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Developmental mechanisms patterning 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, it is characterized by a huge diversity of neuronal phenoptypes, each cortical neuron displaying a specific pattern of connectivity. The mechanisms of formation of cortical networks have direct relevance to several diseases, such as epilepsy and mental disorders, as well as for the development of rationally designed cell therapies for neurological conditions.

The major research goal in our laboratory is to understand the mechanisms controlling the development of the cerebral cortex, from stem cells to neuronal networks, by combining molecular and cellular approaches, both *in vivo* (using mouse transgenesis and in utero electroporation) and *in vitro* (using organotypic assays and embryonic stem cells).

Below are summarized the work achieved thanks to the Funding of the FMRE/GSKE over the past year, providing a link with our previously published work, as well as their perspectives in the future.

1. Multiple roles for ephrin/Eph genes in the development of the cerebral cortex.
Recent work from our group, centered on the analysis of ephrin/Eph compound mutant mice, demonstrated that ephrin/Eph genes are required not only for the control of topographic specificity within the somatosensory area, but also unexpectedly for the *inter-areal* specificity of thalamocortical projections between the motor and the somatosensory systems (Dufour et al., 2003;Egea et al., 2005;Seibt et al., 2003;Vanderhaeghen and Polleux, 2004)

In parallel we generated a novel ephrin gain of function mouse model, enabling ectopic, patterned, expression of ephrin-A5 in the developing cortex. The analysis of this model, combined with our study of ephrin/Eph knock-outs, enabled us to demonstrate an important role for ephrins in the control of brain size, through the unexpected regulation of apoptosis of neural progenitors (Depaepe et al., 2005;Depaepe and Vanderhaeghen, 2005). Our findings suggest that ephrins, like neurotrophins, have evolved as pleiotropic factors that can control very different functions depending on the cellular context, which could have important implications in other aspects of developmental or stem cell biology, and in oncogenesis. We are following up on these observations, focusing on the physiological impact of ephrin-mediated apoptosis in the patterning of different brain regions, and exploring the links between ephrin-dependent signals mediating apoptosis and axon guidance, both in neurons and in neural stem cells. In addition we are taking advantage of our gain of function model to study further the roles of ephrins in the development of neuronal connectivity in the cortex.

2. Mechanisms upstream of ephrins during brain development.
A critical but still unsolved issue of forebrain development concerns the mechanisms responsible for the specification of distinct areal domains in the neocortex (Sur and Rubenstein, 2005). In this context, the early appearance of graded ephrin/Eph expression in

several areas of the cortex (Vanderhaeghen et al., 2000) raises the question of how these gradients of expression are generated and controlled.

A transgenic approach is being used to address this problem. First, we are generating mouse knock-in lines allowing expression of reporter genes (eGFP and PLAP) under the control of all regulatory sequences of ephrin-A5. The construct was completed this past year and is now being targeted to the mouse geneome using ES cells. We will use these mice to study in vitro the modulation of expression of ephrin-A5 in the developing cortex in organotypic cortical cultures. This will allow us to study with great sensitivity and in real time how ephrin-A5 gradients are generated in the cortex and which loco-regional factors control their onset. These ephrin-A5/GFP/PLAP mice will also enable us to search for genes associated with the gradients of ephrins-A5 using differential expression screens.

3. Control of neuronal migration to the striatum and the cerebral cortex.

Work from several laboratories has demonstrated that in rodents most cortical GABAergic interneurons are generated in the ganglionic eminences (GE) in the ventral telencephalon, and subsequently migrate to the cerebral cortex through several streams of tangential migration (Marin and Rubenstein, 2001). Most interneurons destined to the cerebral cortex are generated within the medial GE (MGE), while the lateral GE (LGE) essentially generates neurons destined to the basal ganglia. This dorso-ventral sorting of migrating neurons may have important functional and behavioural consequences, such as the balance of GABAergic innervation in the striatum and cortex.

We have recently set up organotypic assays combined with electroporation-based transfection to dissect the molecular and cellular mechanisms that control the migration of distinct populations of neurons to specific domains of the telencephalon: in this system, GE explants overexpressing eGFP (obtained from transgenic mice or following focal electroporation) are co-cultured with organotypic slices of developing telencephalon and the migration patterns of eGFP+ cells can be monitored over several days. These assays allow to recapitulate several important aspects of tangential migration, such as the dorso-ventral sorting of MGE vs LGE neurons that occurs in vivo. Using these in vitro assays, we have started to study the role of several candidate guidance factors including ephrins in the patterning of the migratory streams in the basal forebrain and cortex. We plan to follow up on these in vitro observations using in utero electroporation and appropriate mouse transgenic models, in order to test for the consequences of the early disruption of migration patterns on cortical function in mature animals.

4. Molecular mechanisms of neuronal specification using ES cell-derived neurons.

The molecular mechanisms of neuronal specification involve region-specific morphogens that play an instructive role to drive progenitors to particular cellular phenotypes. For instance in the the telencephalon, several transcription factors of the Homeodomain and Helix-Loop-Helix families are differentially expressed in distinct domains and cooperate to generate distinct

neuronal types such as pyramidal neurons in the cortex, projection neurons in the striatum or diverse types of cortical and striatal interneurons (Schuurmans and Guillemot, 2002). However the identity of the genes acting downstream and upstream of these transcription factors, as well as the genetic programmes involved in the generation of the huge diversity of cortical projection neurons and interneurons remain largely obscure.

Recently we have implemented a technique to generate large numbers of neural progenitors and neurons from ES cells using adherent monoculture in serum-free media. Interestingly we recently found that in these conditions ES cells can follow a highly stereotyped pattern of specification, that yields to the efficient generation of glutamatergic pyramidal-like neurons that appropriately integrate in postnatal cortex in vitro. Given the many advantages offered by ES cells in terms of genetic manipulation, we are currently optimizing our system in order to use it for functional genetic screens, looking for genes that are associated to, or instructive for, particular neuronal phenotypes. We have also started to implement similar methods to human ES cells to generate stereotyped patterns of pyramidal neuron specification. This could have a major impact for the design of cortical neuron replacement therapies, and in our understanding of human neuronal development. The use of human ES cell models could also be used in conjunction with our gene expression studies performed in human embryonic brain (cf 5).

5. Developmental basis of human-specific features in the cerebral cortex.

Although many aspects of brain development seem to be remarkably conserved throughout evolution, a number of neural features have undergone a considerable divergence in mammals, in particular in the forebrain. We therefore started a project focusing on the human developing brain, trying to reveal what are the specific developmental programmes underlying the emergence of human-specific features in our brain. This project already allowed us to demonstrate for the first time the involvement of ephrin/Eph genes in the development of the human brain, and to propose a new model for the emergence of a binocular visual system during mammalian evolution (Lambot et al., 2005). Currently we focus our efforts on the cerebral cortex, using in situ hybridization and microarray analyses, trying to unravel novel genes specifically involved in human cortical development. This molecular and anatomical approach, together with the exploitation of the ever-increasing data available from human genetics and comparative genomics, and the use of human ES cell-derived neurons (cf 4), should yield important new insight on the developmental basis of the evolution of the human brain and on the mechanisms of neurodevelopmental disorders.

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Interactions between areas investigated using awake monkey fMRI.

In-vivo tract tracing by combining electrical microstimulation and fMRI (EM-fMRI)

In a first series of GSKE-funded experiments, we developed, tested and validated a new method to perform in-vivo tract tracing in awake behaving monkeys.

In order to fully understand neural mechanisms involved in sensory, cognitive or motor task, it is mandatory to know the anatomical and functional connectivity between brain regions that are involved in the processing of these mental operations. Until now, the most reliable method to trace connections between brain regions have been conventional antero-and retrograde tracer techniques in which chemical substances are injected in the brain focus of interest. These substances are incorporated in the neurons and antero- or retrogradely transported along the axons. Recently, improved methods were developed for tracing pathways with enhanced sensitivity and even transneuronally, for example using neurotropic viral tracers (1, 2). Unfortunately, these methods require that the animals are sacrificed in order to visualize the transported tracers microscopically and cannot be used for follow-up electrophysiological or inactivation studies. Recently, however, there have been exciting advances in methods for tracing connections in vivo using 1) DTI, which allows tracking of white matter fiber trajectories even in humans (3), and 2) using focally injected MRI-visible susceptibility agents (4). Although both approaches are very promising, questions remain about their sensitivity, precision and accuracy. For example, several well-know difficulties exist for all DTI-based tractography approaches, such as the mathematically intricate problem to resolve 'kissing' and 'crossing' of fibers within the individual voxels. High angular resolution diffusion imaging (HARDI) based approaches (5) might resolve some of these problems, yet a maybe even more problematic issue with DTI-based tractography is that the algorithms used to 'define' the course of a track from one voxels to its neighbors are NOT model-free. The second in-vivo tractography method using MRI-visible contrast agents, still suffers heavily from local diffusion and/or blooming effects at the site of injection as well as the target zones. Also, the sensitivity of DTI-based tractography and of MRI-visible tract tracing still needs to be assessed and validated. Finally, the combination of human functional imaging techniques with transcranial magnetic stimulation also showed promising, though spatially crude results to resolve 'functional' connectivity in the human (6, 7).

Inspired by the latter studies tested the hypothesis that the novel combination of EM-fMRI in awake animals allows us to identify functionally connected regions in-vivo, with higher sensitivity, precision and accuracy compared to the aforementioned in-vivo techniques. Intracortical microstimulation is particularly powerful as it allows to increase the output signal of a group of neurons with reasonable spatial (e.g. at columnar level) and temporal resolution (i.e. at millisecond level) and to demonstrate causal links between neural activity and specific cognitive functions.

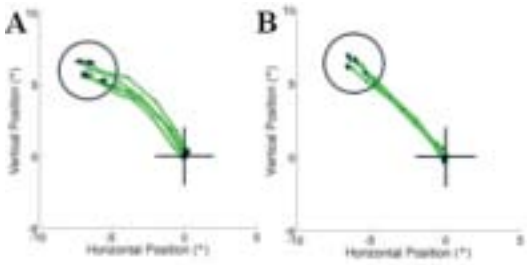

Fig. 1: Eye traces of evoked saccades recorded in MR scanner; (A) and (B) collected 6 months apart, stimulating same electrode

Because it is well-known that microstimulation can evoke ortho-and antidrome responses in neurons connected with the site of microstimulation (8-10), it is reasonable to assume that this should also lead to increased fMRI activity in the areas connected with the site of stimulation. Recently, Tolias and coworkers demonstrated the feasibility of this approach in anesthetized monkeys (11). In this study large currents were needed to evoke functional effects in only a very limited number of areas. We chose to test the EM-fMRI approach in awake monkeys with full-brain coverage. In the awake preparation much less currents are needed to evoke behavioral (and likely functional MRI effects), i.e. 1-2 orders of magnitude lower than those used in the Logothetis' study. Hence we expect much less local spread compared to the study by Tolias et al.. Moreover, to evaluate this technique, we studied the functional MRI effects during focal microstimulation of the FEF.

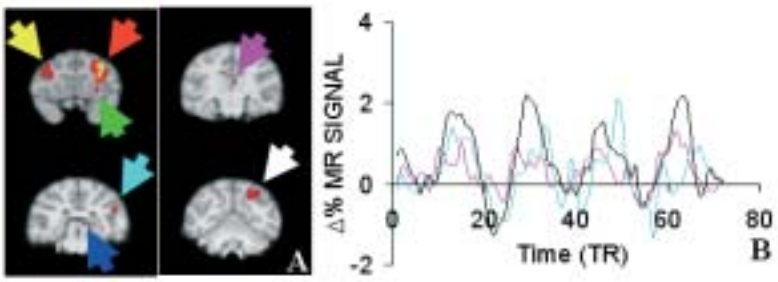

Fig. 2: (A) Coronal slices showing fMRI activity induced by FEF microstimulation ($p < 0.05$, corrected), (B) Time-courses of % Change in MR Signal for 3 of the areas identified; Labels: Red = ipsilateral FEF, Yellow = contralateral FEF, Green = claustrum, Pink = thalamus, Cyan = STP, Blue = S. Colliculus, White / Black = LIP

The advantages to validate and optimize the EM-fMRI approach in the FEF are four-fold: 1) Clear-cut behavioral responses (saccades) can be evoked by microstimulating small groups of FEF neurons, giving direct feedback about the actual location of the respective electrodes. 2) The current needed to evoke a behavioral response can be accurately titrated. During the EM-fMRI experiment sub-threshold currents can be employed in order to circumvent motor- and/or visually-related (e.g. retinal slip) changes in fMRI activity. 3) The anatomical connections of the FEF are very well studied (12-17) and the results of these traditional tractography experiments can immediately serve as ground-truth to validate the proposed EM-fMRI method. 4) The FEF is topographically nicely organized with a representation of small saccades more ventrolateral and larger saccades more medio-dorsal (13). Also these subdivisions of the FEF are known to be connected with largely different portions of extrastriate visual cortex: dorsal stream areas are connected with the large-saccadic region, while ventral stream areas are more connected with the more lateral small-saccadic region within the FEF (12). Hence, we predict a slightly different pattern of EM-fMRI defined functional connections from the respective subregions of the FEF.

Methods

Two monkeys were equipped with a magnet-compatible headset and twenty-five micron thick micro-electrodes were inserted in the FEF. We used standard contrast-agent enhanced awake monkey fMRI techniques (for details see (18-27)) at 3T. Monkeys fixated to a fixation spot (within a 1 X 1 degree fixation window) presented on a screen 50 cm in front of their eyes (in the absence of any visual stimulus). Prior each fMRI experiment, we determined inside the scanner the individual movement fields (MFs, i.e. amplitude and direction of the evoked saccades) of the neurons near the tips of 1 to 4 electrodes that were used in the subsequent fMRI experiment (typical block design). This test also revealed the current threshold needed to evoke saccades from the individual electrodes. In each experiment, we selected 1-4 electrodes that evoke a wide variety of saccades (i.e. an as wide range of amplitudes and directions possible).

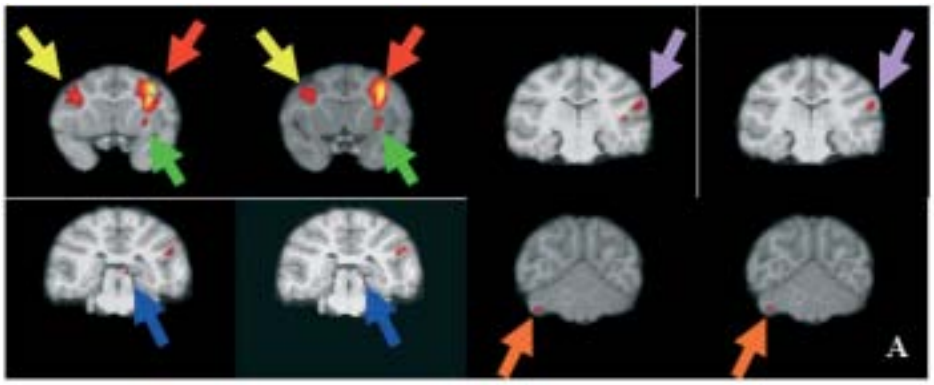

Fig. 3: Coronal slices showing FEF-microstimulation dependent modulation of fMRI activity ($p<0.05$, corrected) Test-retest (experiments one month separated in time

In a first series of EM-fMRI experiments, we stimulated the FEF (at subthreshold current levels) while the monkey fixated to a fixation spot in the absence of visual stimulation. Comparison of fMRI activity between epochs with and without microstimulation revealed robust, focal, and repeatable fMRI activity at distant locations, known to be anatomically connected to the FEF (12-17, 28). Here we illustrate only a few examples of functionally connected regions with the microstimulated FEF. Fig. 2 shows 4 coronal slices with increased fMRI activity ($P<0.05$ corrected) caused by microstimulating the FEF. The corresponding fMR timecourses are indicated in panel 2B. The next figure (Fig. 3) illustrates the reproducibility of the microstimulation induced changes in fMRI activity at distant sites from the microstimulation. Two experiments were performed more than 1 month apart from each other and revealed near-identical results proving the high precision of the technique. Notice that even for a small number of co-activated voxels (e.g. within the colliculus superior and cerebellum), the results are remarkably-well reproducible, which is also indicative for the spatial sensitivity of the EM-fMRI technique. The next figure, on the other, hand shows the accuracy of our EM-fMRI results. Indeed, microstimulating the FEF revealed virtually identical 'in-vivo' functional connectivity foci compared to existing anatomical tract tracing data from the FEF (12-17, 28) as illustrated for a few examples in Fig. 4.

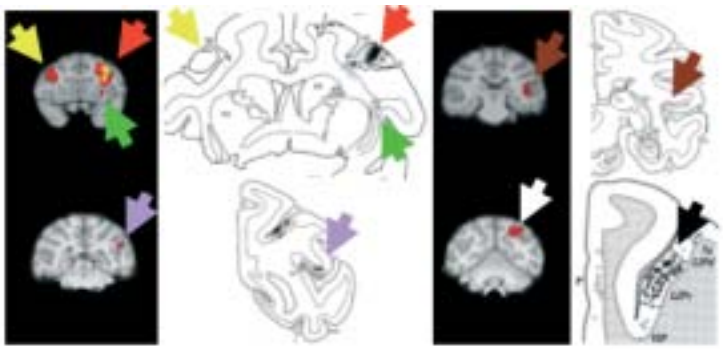

Fig. 4: Coronal slices comparing areas of fMRI activity induced by microstimulation ($p < 0.05$, corrected) with slices from anatomical tract tracing experiments (LIP - Schall et al., 1995, rest - Huerta et al., 1987). Labels: as above, Brown = MSTv

(C)overt attention

In a second series of experiments, we began to study the functional role of connections arising in the FEF using the EM-fMRI technique. As suggested before, the FEF might play an important role as attentional top-down source to modulate incoming sensory information. We started a series of experiments (only the first few will be discussed in this progress report), in which we try to mimick spatial attention-like modulations of neuronal activity by artificially increasing the output of the FEF. The long-term goal of this study is to compare in a quantitative manner spatial-attention induced modulations of functional activity with that induced by artificially increasing the output of the FEF, as well as the behavioral consequences of both 'interventions'. First I will discuss the background for these studies.

Although we are continuously bombarded with an enormous amount of visual information, our visual system is not equipped to process all this incoming sensory information equally well. One way to reduce the abundance of incoming visual information for detailed visual processing, is to attend, at any given time, to a particular location in space. Contemporary biased competition models (29-31) proposed that there is a competition for neural resources, according to which the competition among stimuli for neural representations can be biased in several ways. Those stimuli that win the competition from their distractor stimuli 'survive' and will have further access to higher order neural mechanisms where perceptual processes are linked with those generating goal-directed actions (e.g. systems involved in memory, decision-making, generating motor plans, etc..) (29, 32, 33).

There is ample evidence from electrophysiology, functional imaging and psychophysics that visual representations and perception are enhanced at both overtly and covertly attended locations. For example, neurons in extrastriate (and even striate) visual cortex representing objects at attended locations increase their firing rate (e.g. (29, 32, 34-43). Similar results were obtained in human subjects using neuroimaging techniques. For example spatial attention leads to stronger activations in extrastriate cortex in the hemisphere contralateral to the attended side of a stimulus array (44). These results were later refined using fMRI: exactly at the retinotopic representation of attended stimuli one observed attention-dependent modulations of activity (45-47).

Source signals for attentional modulation?

Thus neurons in extrastriate and even striate visual cortex can significantly modulate their firing rate, depending on the direction of attention of the subject with respect to the receptive

field of the neuron. Furthermore, a clear correlation has been suggested between such 'increased' neuronal activity at attended locations and corresponding benefits at behavioral level. However, the neural mechanisms that give rise to these effects are poorly understood. How do neurons in visual cortex 'know' which location is attended? Two possible mechanisms have been suggested: a bottom-up mechanism in which salient stimuli in the visual scene automatically 'attract' attention without the need of endogenous signals. Secondly, it is widely recognized that voluntary and hence endogenous attentional 'source' signals emerge in higher level areas within a fronto-parietal network. Feedback signals from these areas to lower level sensory areas are thought to modulate incoming sensory information through feedback and lateral connections.

Premotor theory of spatial attention

Under normal circumstances, the direction of attention is usually aligned with the direction of gaze because the oculomotor system continuously shifts the most relevant information of a visual scene on the fovea, that portion of the retina with the highest acuity. However, sometimes, the direction of gaze can be disengaged from the direction of attention, a process referred to as covert attention -as opposed to the 'normal' overt attention conditions (48, 49). A body of evidence has suggested that the neuronal mechanisms by which the processing of relevant information is 'enhanced' under covert and overt attention conditions might be largely similar to that involved in reorienting the gaze. Specifically, Rizzolati and co-workers suggested that the neural mechanisms of covert spatial attention are strictly linked to those controlling eye movements (48, 50-58). The basic rationale of this 'premotor theory of attention' is that when a 'cue' is presented to attend to a particular location -be it exogenously or endogenously-, an oculomotor program is built up, which, if executed, brings the fovea onto the to-be-attended target.

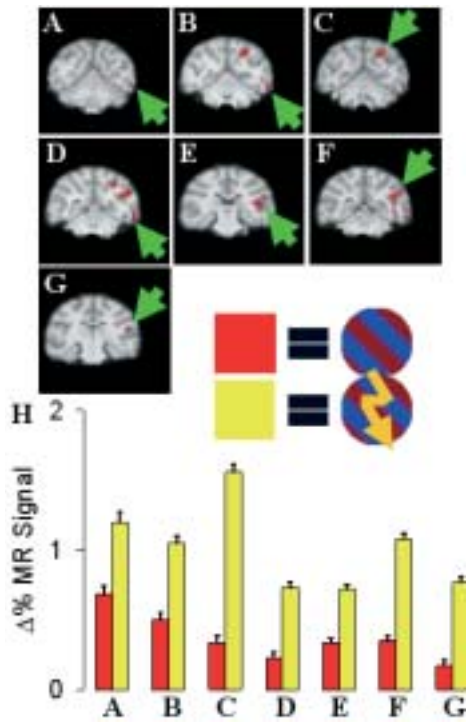

Fig. 4: Coronal slices showing visually-driven areas, in which epochs of combined visual stimulation and microstimulation produced greater fMRI activity than epochs of visual stimulation only ($p<0.001$ uncorrected, analysis limited to visually-driven areas only); Arrows label (A) V2, (B) V4, (C) LIP, (D) TEO, (E) MSTv, (F) MSTd & (G) STP; (H) % Change in MR signal between the two epochs (baseline activity is fixation point only epoch)

Causal interactions between fronto-parietal 'source' areas and visual cortical 'site' areas?

Although both the functional imaging studies and neuropsychological evidence highly suggests that feedback signals from the fronto-parietal cortex interact with sensory signals in striate and extrastriate visual cortex, it has been difficult to prove a causal link between activity in the frontal (or parietal) cortex and spatial attention.

The most elegant evidence to date, however, showing a direct causal link between artificially increased output of a frontal area and improved performance levels at attended spatial locations was provided by Moore and colleagues (57). These authors electrically microstimulated the frontal-eye-fields (FEF) in monkeys, a prefrontal cortical area involved in the generation of purposeful saccadic eye-movements, and observed decreased contrast detection thresholds at retinotopic locations corresponding to the stimulated movement fields (MFs) of the FEF. This artificially induced behavioral enhancement could not be obtained when stimuli were presented at positions not corresponding to the stimulated MFs, indicating 1) the spatial specificity of the results, and 2) that the results cannot be explained by general increases in arousal.

In a second set of experiments, the same group was also able to modulate visually driven activity of V4 neurons, when the corresponding FEF-MFs were stimulated at a subthreshold level (58). These microstimulation effects were quantitatively similar to attentional modulation of neuronal activity as observed previously within this cortical visual area (32, 33, 38, 39). Notably, the magnitude of the microstimulation effects on V4 neurons depended on the effectiveness of the receptive field stimuli as well as the presence of competing stimuli outside the receptive field. Furthermore, NO modulation of V4 activity was observed in the absence of a stimulus, indicating a lack of change in background activity.

In our second series of experiments we elaborated on these well-designed series of microstimulation experiments to show a causal relationship between artificial increased output of specific sectors of the FEF and visual representations in the visual cortex. As mentioned above, and if the FEF is indeed one of the top-down sources that can modulate visually-driven activity in an attention-dependent manner, one would predict shifts in baseline activity in visual cortex after FEF microstimulation (as illustrated in Figs. 2 and 3). Also, attention-dependent modulation of activity has been observed throughout visual cortex, with increasingly larger modulations as one moves away, at least in synaptic dimensions, from the

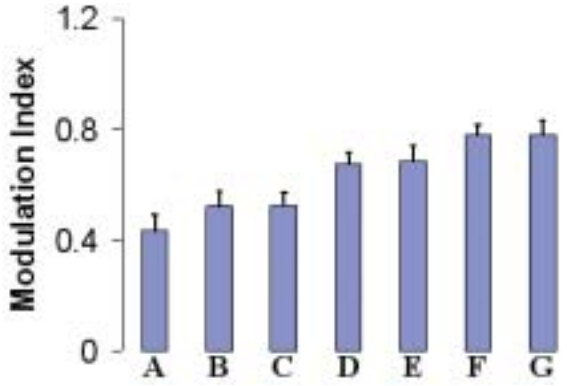

Fig. 5: Modulation Index. Labels: A = V2, B = V4, C = MSTv, D = MSTd, E = TEO, F = LIP, G = STP

retina. Using the proposed EM-fMRI approach, we tested for EM-dependent modulations of visually-driven activity throughout the brain. In early visual cortex, the modulatory microstimulation effects should be retinotopic specific (i.e. match the MFs of the stimulated FEF).

This was tested by microstimulating FEF-movement fields that overlap with a visual stimulus and investigate whether or not visually driven activity can be modulated by the increased output of the FEF neurons with movement fields overlapping with the retinotopic position of the stimulus. The results from this experiment are illustrated for a few examples in Fig. 4. A final prediction from the spatial attention literature that we tested was that this microstimulation-induced modulation of visually driven activity increases in function of synaptic distance from the retina (see Fig. 5). In this figure we show the modulation index [(visual + electrical stimulation) - (visual stimulation)]/ (visual + electrical stimulation) for several areas showing a modulation of visually driven activity at $P < 0.05$ corrected for multiple comparisons.

In conclusion, our preliminary studies clearly showed 1) the feasibility of the EM-fMRI technique to perform in-vivo tract-tracing experiments. Results of such studies can serve as ground-truth to validate DTI-based tractography approaches which can also be used in humans. 2) As mentioned above, the EM-fMRI approach will allow us to directly test a number of predictions from the spatial-attention literature which should hold true in case the FEF is one of the top-down sources for attentional modulations of sensory driven activity. For example, the attention literature provides clear-cut predictions for the modulation of activity when 1 (shift in contrast-gain function) or multiple stimuli (attenuation of mutal suppression of activity) are presented within the MFs of stimulated FEF neurons. 3) The EM-fMRI technique opens the possibilities to perform invasive follow up studies in the same animals (e.g. combined (ir)reversible deactivation and, electrophysiological recordings). Such a combination of techniques is extremely valuable to investigate how information is being transformed in a distributed network of co-activated regions during a wide variety of mental operations.

We will start pursuing these questions during the next two years of this grant.

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Progress Report of the Research Group of

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The human 5-HT7 receptor: a new link and potential therapeutic target for Alzheimer's Disease?

1. Introduction: context and aim

Serotonin (5-hydroxytryptamine or 5-HT) is an important neurotransmitter involved in diverse physiological processes such as sleep, sexual behaviour, food intake, mood regulation and certain cardiovascular and gastro-intestinal functions. Pathological conditions associated with a dysfunction of the serotonergic neurotransmission are numerous. These include depression, schizophrenia, 'generalized anxiety disorder' (GAD), 'obsessive-compulsive disorder' (OCD), migraine, hypertension and eating disorders (Jones and Blackburn 2002). This high diversity of physiological effects is correlated with a large variety of 5-HT receptors. Until now, 14 different 5-HT receptor genes have been identified, which, with exception of the 5-HT3 receptors, all belong to the family of 'G protein-coupled receptors' (GPCRs) (Hoyer et al. 2002). This diversity is even more amplified by post-genomic modifications such as alternative splicing and editing of the mRNA.

The most recently identified serotonin receptor is the 5-HT7 receptor (Vanhoenacker et al. 2000). Recent studies suggest involvement of this receptor subtype in thermoregulation, circadian rhythm, REM sleep, migraine, depression, hippocampal signalisation, memory and endocrine regulation (reviewed in (Thomas and Hagan 2004)). Alternative splicing of the 5-HT7 mRNA gives rise to different isoforms, namely 5-HT7(a), (b), (c), (e) in rat and 5-HT7(a), (b), (d) in human (Heidmann et al. 1997; Heidmann et al. 1998; Liu et al. 2001). These splice variants differ in the length of their intracellular C-terminal tail and in the number of potential phosphorylation sites within this region. This suggests variation in signalisation and regulation. To further investigate these potential differences, we decided to search for proteins interacting with these different C-terminal regions. Therefore, we performed a yeast two-hybrid screening with the C-terminal end of the human 5-HT7(a) receptor as a bait and an adult-human brain cDNA library as prey. This led to the identification of several interesting possible interaction partners, of which the human β-Amyloid Precursor-like Protein-1 (APLP1) was one. This interaction might indicate an innovative link between the 5-HT7 receptor and Alzheimer's Disease.

APLP1 belongs, together with APLP2 (amyloid precursor-like protein 2) and APP (Amyloid Precursor Protein), to the 'APP family'. These proteins are very homologous and are all type I, integral membrane proteins with a large glycosylated extracellular region (N-terminal), one transmembrane helix and a short C-terminal cytoplasmic tail.

These proteins are all processed by proteases. First, they are cleaved in their extracellular part by the α- or β-secretase resulting in a soluble extracellular domain (sAPPα/β or sAPLPα/β) and an intermediate membrane-bound fragment (C83 and C99, respectively). The latter is then further processed by the γ-secretase. Subsequent processing of APP by the β-secretase and the γ-secretase results in the secretion of the amyloid β-peptide (De Strooper and Annaert 2000; Annaert and De Strooper 2002). This is the most prominent component of the extracellular plaques, found in the brain tissue of Alzheimer patients. Two types of protein aggregates in the

brain characterize Alzheimer's Disease: the intracellular neurofibrillary tangles (NFT), consisting of hyperphosphorylated tau, and the extracellular amyloid plaques, containing mainly amyloid-β peptide fibrils. Alzheimer's Disease is a progressive incurable neurodegenerative disorder associated with impairments in cognition and memory. It is the major cause of dementia in older people, with ~10% of those over the age of 65 and 50% of those over 85 affected. By 2050, the population over 65 years of age will approximately double. This translates to well over 20 million patients with AD in the United States and Europe.

2. Research: current status and prospectives

A. Interaction studies

In order to validate the interaction observed in the yeast two-hybrid experiments, we decided to first investigate whether both proteins interact in eukaryotic cells. After transient transfection in COS cells we were able to demonstrate co-immunoprecipitation of APP (cDNA was a kind gift from Dr. B. De Strooper, Center for Human Genetics, University of Leuven, Belgium) and APLP1 with all three 5-HT7 receptor isoforms (figure 1A and 1B). We also could co-immunoprecipitate APLP2 with the 5-HT7(a) and (d) isoform, but not with the 5-HT7(b) receptor. This was due to a rather low expression level of the latter and therefore this experiment will be repeated and optimised in the near future (figure 1C). These results confirm the interaction seen in the yeast two-hybrid experiment and seem to indicate that the interaction holds true for all three 5-HT7 receptor variants and all members of the APP-family.

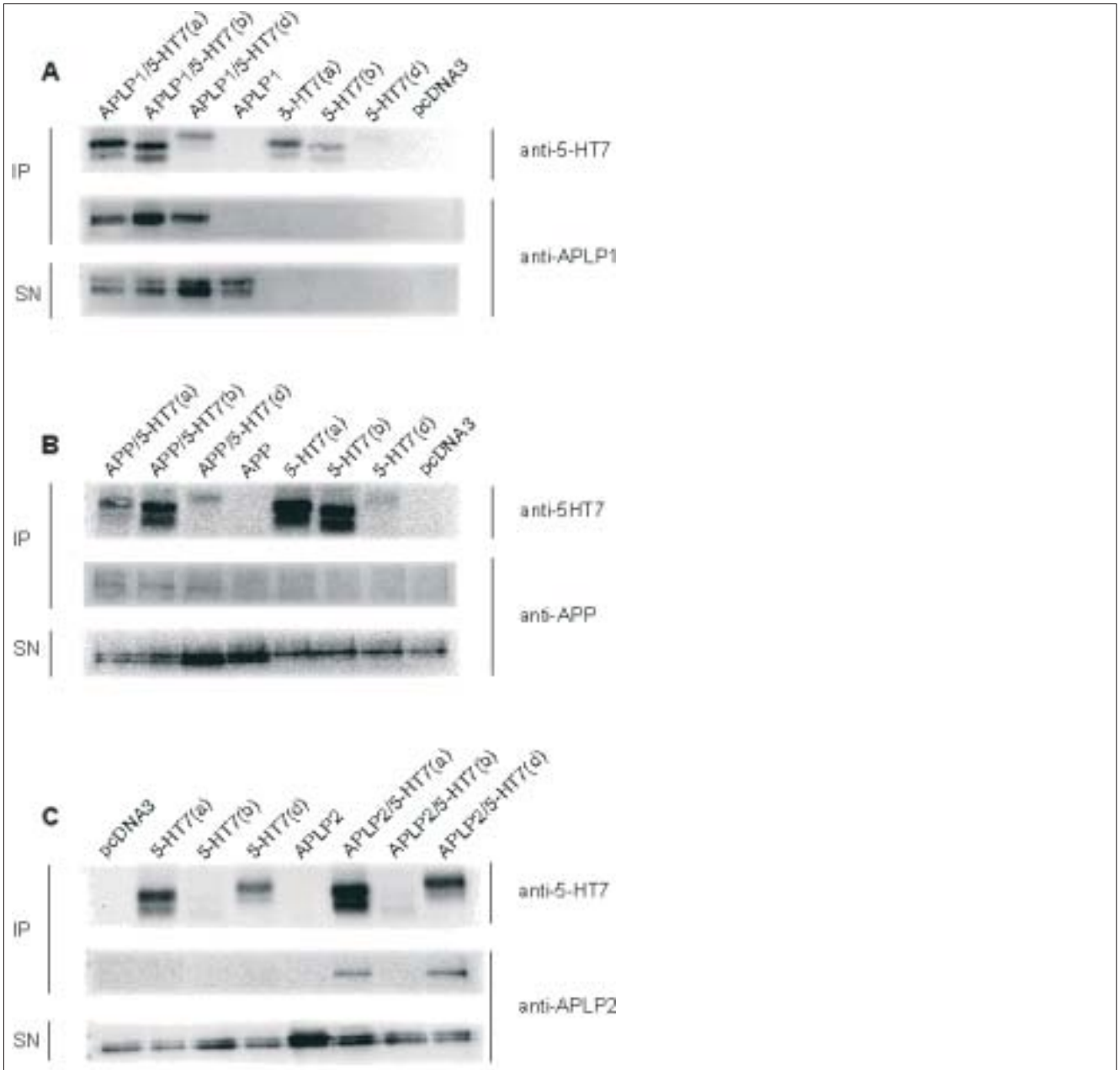

Figure 1: Co-immunoprecipitation experiments between the 5-HT7 receptor isoforms and the 'APP family of proteins'.
COS cells were transiently transfected with the three 5-HT7 receptor isoforms and human APLP1 (A)/APP (B)/APLP2 (C), alone or in combination. Immunoprecipitation was performed with a goat antibody directed against the N-terminus of the 5-HT7 receptor (common to all isoforms). The immunoprecipitated proteins were revealed with anti-APLP1 (anti-CT11); anti-APP (gift from Dr. B. De Strooper); anti-APLP2 (gift from Dr. D. Walsh) or a rabbit anti-5-HT7. SN indicates the amount of APLP1/APP/APLP2 present in each sample before immunoprecipitation. IP indicates the immunoprecipitated proteins. pcDNA3 represents the vector without insert.

In order to verify that these interactions are not due to transient overexpression of both proteins we made use of CHO cells, stably expressing APP (gift from Drs. D. Walsh, Conway Institute, Dublin, Ireland and D. Selkoe, Center for Neurologic Diseases, Boston, Massachussetts). After transient expression of the 5-HT7(a) receptor in these cells, we could also co-immunoprecipitate APP with the 5-HT7(a) receptor (figure 2).

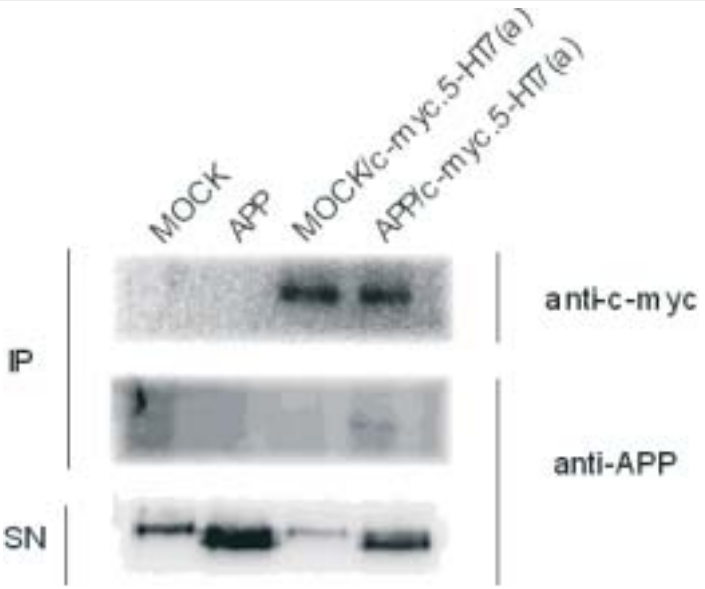

Figure 2: Co-immunoprecipitation between the c-myc.5-HT7(a) receptor and APP in CHO cells, stably transfected with APP.
CHO cells, stably transfected with pcDNA3 (MOCK) or APP were either left untreated or transiently transfected with c-myc.5-HT7(a). Immunoprecipitation was performed with an anti-c-myc antibody (4A6). The immunoprecipitated proteins were revealed with either anti-APP or anti-c-myc (revealing the tagged receptor). IP indicates the immunoprecipitated proteins. SN indicates the amount of APP present in each sample before immunoprecipitation.

This result confirms the interaction seen in COS cells and thus suggests a direct interaction between the APP proteins and the 5-HT7 receptors. However, the 5-HT7 receptors and the APP proteins are both transmembrane proteins and thus firmly attached to the plasma membrane of eukaryotic cells. This implicates the possibility that these proteins co-immunoprecipitate without direct interaction, but rather as a consequence of their anchorage in the plasmamembrane. To further confirm that these proteins are genuine interaction partners, we decided to follow an in vitro GST pulldown approach. For this, we first produced ^{35}S-Methionine labelled APP and APLP2 by in vitro transcription/translation. This material was then incubated in vitro with the C-terminus of the 5-HT7(a) receptor attached to a GST tag and to the GST protein alone (which serves as a negative control). After immunoprecipitation of the GST proteins with glutathion beads, we detected a clear ^{35}S-signal for APP and APLP2 on the beads with the C-terminus of the 5-HT7(a) receptor, but not on the beads with the GST protein alone (figure 3). This experiment convincingly demonstrates that APP and APLP2 interact directly and specifically with the C-terminus of the 5-HT7(a) receptor in vitro. Similar experiments for APLP1 are currently ongoing. All together, these data strongly suggest direct interaction between the APP proteins and the 5-HT7 receptors.

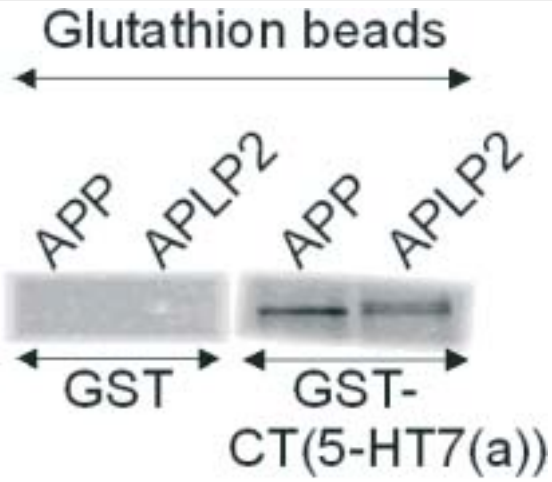

Figure 3: GST pulldown of GST-CT(5-HT7(a)) with APP and APLP1.
APP and APLP1 were produced by in vitro transcription/translation in the presence of ^{35}S methionine. These proteins were then added to the GST-CT(5-HT7(a)) fusion protein and to GST protein (as a negative control). After immunoprecipitation with glutathion beads, proteins were separated by SDS-PAGE and visualised using a PhosphorImager.

In the yeast two-hybrid screening, we used the intracellular part of the 5-HT7(a) receptor as a bait. Therefore, it is reasonable to assume that the interaction with APLP1 occurs via its intracellular C-terminal part. To confirm this, we performed a co-immunoprecipitation experiment in 293T cells between the 5-HT7(a) receptor and two different C-terminal deletion mutants of APLP1, namely (C51 and C60), which were kindly provided by Dr. D. Walsh. These constructs correspond with the intracellular fragments formed after cleavage by the γ-secretase. Unfortunately, we were not able to co-immunoprecipitate these fragments with the 5-HT7(a) receptor (figure 4). This could be due to a decreased stability of these fragments and the fact that these APLP1 fragments are, in contrast to the mature APLP1 and the 5-HT7 receptor, not membrane-bound and thus possibly not located/available at the plasmamembrane. It has been shown that the cleaved intracellular fragments of the APP proteins translocate to the nucleus together with Fe65, resulting in the transcription of certain genes (Scheinfeld et al. 2002; Walsh et al. 2003). To avoid this possible topological problem 'in vivo', we are currently using the 'in vitro' GST pulldown approach (see above) to further investigate this aspect.

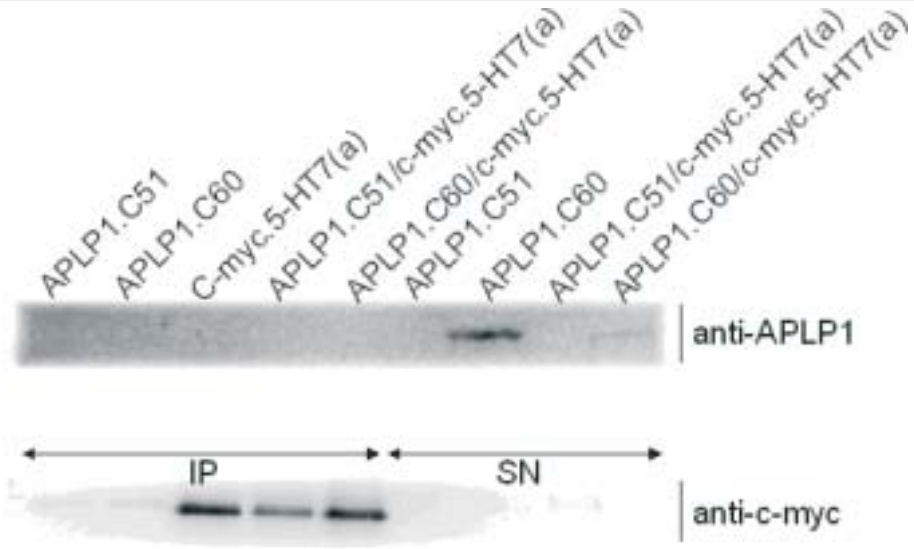

Figure 4: Co-immunoprecipitation between the c-myc.5-HT7(a) receptor and APLP1.C51/C60 in 293T cells.
293T cells were transiently transfected with the c-myc.5-HT7(a) receptor and the two C-terminal fragments of APLP1 (C51 and C60), alone or in combination. Immunoprecipitation was performed with an anti-c-myc antibody (4A6). The immuno-precipitated proteins were revealed with either anti-APLP1 (anti-CT11) or anti-c-myc (revealing the tagged receptor). IP indicates the immunoprecipitated proteins. SN indicates the amount of APP present in each sample before immunoprecipitation.

Finally, we will also investigate whether the interaction is restricted to the 5-HT7 receptors. In this respect, we will certainly investigate potential interaction with the 5-HT4, 5-HT2A and 5-HT2C receptors as it has been shown that stimulation of these receptors influences the proteolytic processing of APP, although no direct interaction was reported (see section B). The cDNAs and cell lines inducibly expressing these receptors are available within the group (Vanhoenacker et al. 1997; Vanhoenacker et al. 1999; Van Craenenbroeck et al. 2001).

B. Does activation of the 5-HT7 receptor leads to secretion of sAPPα/sAPPβ?

It has been documented that stimulation of certain 5-HT receptors influences the proteolytic processing of APP. Activation of the 5-HT4, 5-HT2A and 5-HT2C receptors leads to an increased secretion of the non-amyloidogenic sAPPα fragment (Lezoualc'h and Robert 2003; Nitsch et al. 1996). Therefore, we found it worthwhile to investigate whether this also holds true for the 5-HT7 receptor. In order to study this in physiological relevant conditions, we tested a number of "neuronal cell lines" for endogenous expression of both the 'APP family of proteins' and the 5-HT7 receptor. An additional criterion was reasonable high transfection efficiency.

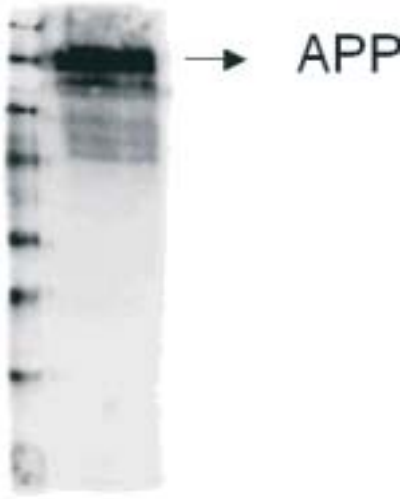

Fig. 5: Endogenous expression of APP in the mouse neuroblastoma cell line, HT22.

The mouse neuroblastoma cell line HT22 not only appeared to exhibit a high endogenous expression of APP (figure 5), but also showed satisfying transfection efficiency after transient transfection using the calcium phosphate method. By RT-PCR, we could also demonstrate the presence of 5-HT7 specific mRNA (data not shown) and we are currently optimising the conditions to reveal the presence of the 5-HT7 receptor at the protein level. In the mean time, we are investigating whether induction of the 5-HT7 receptor (upon overexpression after transient transfection) leads to an increased secretion of APP fragments. If this is the case, then this pathway will certainly be further analyzed. First, we will study the involvement of Epac, as it has recently been shown that 5-HT7 receptor-mediated Erk1/2-activation, as well as 5-HT4 receptor-mediated APP proteolysis are both dependent on Epac activity (Lin et al. 2003; Maillet et al. 2003). For this, we will make use of dominant negative and constitutively active mutants of Epac. Secondly, we will also study the release of the intracellular domain of APP (AICD) after intramembranous cleavage by the γ-secretase. This AICD fragment is rapidly degraded, unless it becomes stabilized through interaction with another protein, such as Fe65. The Fe65/AICD complex has been shown to translocate to the nucleus. There it interacts with the nuclear histon acetylase, Tip60 (Von Rotz et al. 2004), and can activate the transcription of certain genes (e.g. KAI-1 and GSK3β). If an increase in the intracellular release of AICD would be observed, then interaction with Fe65, nuclear translocation and Tip60-mediated chromatin decondensation will be investigated. For the latter, we will use techniques such as nucleosome positioning and chromatin immunoprecipitation assays, for which the expertise is already present within the research unit (Vermeulen et al. 2003).

C. Role in receptor transport

An aspect of normal APP physiology is the role of APP in the axonal transport of vesicles. Therefore, it is possible that the 'APP family of proteins' has an essential role in the transport of the 5-HT7 receptor within neurons. To study this, we will make use of our available immortalized neuronal cell lines or of primary neuronal cell cultures. For some of these cell

lines we could already demonstrated the presence of APP, APLP1 and the 5-HT7 receptor by indirect immunofluorescence (data not shown). By comparing the trafficking of wildtype receptors with receptors in which the APP-interacting domain has been deleted, we should be able to study whether the interaction is necessary for a correct trafficking of the receptor. We also have GFP-tagged versions of the different receptor isoforms available which will allow us to study this process in 'real time'.

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Progress Report of the Research Group of

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Coding of action categories in primate cortex.

We have completed a single cell study on the effect of adaptation on the responses and selectivity of macaque inferior temporal neurons. The latter study is not only important for understanding the effects of a preceding stimulus on the responses to a stimulus - relevant for the coding of dynamic stimuli (see below) - but is also relevant for the interpretation of the fMRI adaptation technique to infer neuronal selectivities. We have also started with the planned study on the coding of action categories by macaque temporal neurons, developing stimuli and doing recordings in two passively fixating monkeys.

1. Single cell investigation of fMRI adaptation: changes in responses of inferior temporal neurons to sequential presentations of static, complex visual images.

fMRI adaptation is becoming a popular tool to infer the tuning for stimulus parameters of single neurons. However, inferring neuronal tuning from stimulus-dependent adaptation of fMRI activation is based on several assumptions. Apart from issues related to the relation between BOLD responses and neuronal responses, a critical assumption is that the neuronal selectivity and the stimulus selectivity of the adaptation effect correlate. Thus, if a neuron responds to both stimuli A and B, but not to C, the fMRI adaptation paradigm assumes that adapting the neuron to B will produce a similar response decrease to a subsequent presentation of A than when repeating A, but adapting to C will produce no response reduction for A. We have tested this assumption by single cell (extracellular, action potential) recordings in the anterior inferior temporal (IT) cortex of 2 alert, fixating monkeys. Critically, we demonstrated in an earlier monkey fMRI study (Sawamura et al., J. Neuroscience, 2005), in which one of the 3 animals was the same as one subject in the present single cell study, that the region we recorded from shows fMRI adaptation. As expected, we found that repetition of an identical stimulus (images of objects) suppresses the response of IT neurons. This adaptation was the greatest for the first stimulus repetition. Repetition had a divisive effect on the response strength, with the amount of adaptation varying among neurons. However, in disagreement with the main underlying assumption of the fMRI adaptation paradigm, the neurons showed much less adaptation when two different images (A and B) to which the neuron responded similarly were presented in succession (BA sequence) then when the same images were repeated (AA sequence). Presentation of an image to which the neuron did not respond (C) produced no decrement in response to a successively shown stimulus to which the neuron did respond (CA sequence). The weak adaptation for a sequence of two different stimuli to which the neuron responds was found in two different adaptation paradigms and for different stimulus durations and interstimulus intervals.

Further analysis showed that the assumption that the same tuning function underlies the amount of adaptation and the neuron's tuning (Piazza et al., Neuron, 2004) does not hold in IT. In fact, using adaptation as an index of selectivity would overestimate the response selectivity of that neuron, since, depending on the paradigm, about 50 to 80% difference (instead of the predicted 0%) in the degree of adaptation was present between a BA and AA sequence when A and B produced equal responses in the neuron.

These single cell results caution against an inference of tuning properties from fMRI adaptation data. More general, they suggest that adaptation, at least in IT, does not result from action potential generation dependent mechanisms ("fatigue" like effects) but more likely reflects local, synaptic changes or adaptation of input neurons. Thus, the degree of transfer of adaptation from one stimulus to another may not depend on how strongly the neuron responds to these stimuli (its tuning), but on the number of common synapses (or afferents) for the two stimuli.

This study has been published in Neuron (Sawamura et al., Neuron, 2006; also see a preview comment on this study by K. Grill-Spector in the same issue of Neuron).

2. Tuning of single macaque temporal neurons for visual actions.

The recognition of biological movements is extremely important for reproductive success and survival. Indeed, proper action recognition is essential for normal sexual and social behavior. Single cell work in non human primates as well as lesion studies and functional imaging in humans have suggested that the Superior Temporal Sulcus (STS) in the temporal lobe, the parietal cortex, and frontal areas are important for action recognition. The present study aims at a detailed understanding of the coding of dynamic images of actions in the temporal and frontal cortex. As a first step, we studied the responses of macaque temporal cortical neurons to a parameterized set of dynamic visual images of actions while the animals (two monkey subjects) were passively fixating and were not required to categorize the different actions. This enables us to test the neurons using a relatively wide variety of images and control stimuli, which is difficult in animals performing a discrimination of the stimuli.

Little is currently known about the visual analysis of action stimuli. Oram and Perrett (Journal of Neurophysiology, 1996) showed that neurons in the macaque STS can be selective for the direction of a walking person and at least in some neurons this selectivity is due to motion input since the neurons respond differently to the same person walking forward and backward (the "snapshots" being similar in this case). An interesting framework to study the visual analysis of actions is provided by Giese and Poggio (Nature Reviews Neuroscience, 2003). They propose a parallel, independent action processing in a form and motion pathway. In the form pathway, local form analysis is followed by a representation of individual snapshots of the action sequence (i.e. a sample of single frames defining the action sequence). The action is then coded by means of a temporal integration of succeeding snapshots. In the motion pathway, a similar processing sequence is proposed: local motion analysis, motion analysis of single moments of the action sequence ("snapshot motion") and finally integration of the temporal sequence of the different "snapshot motions" that define the action. In addition to determining the tuning of temporal neurons to a parameterized set of actions, we wanted to seek evidence for "snapshot", "snapshot motion", and action neurons in temporal cortex using well-controlled, relatively simple action stimuli. The presence of these neurons in temporal cortical regions would indicate a convergence of the form and motion pathway for action processing in temporal cortex, likely in the rostral STS.

In collaboration with Dr. F. Pollick (Univ. Glasgow, UK), we developed a novel stimulus set

consisting of three prototypical actions (throwing of an object, lifting of an object and knocking on a door) and their blends. The blending algorithm provided actions that consisted of mixtures of the three prototypes using different weights (20% steps) of each prototype (e.g. 0% throw, 80% lift, 20% knock; 20% throw, 60% lift, 20% knock, etc). This parameterized set of actions can be represented as a triangle with the prototypical actions at its corners and the different blends in between. The blending operation results in smooth transitions between the different action stimuli and allows a measurement of the tuning to the action stimuli in this action space. The action images were rendered as stick figures and point light displays. In the latter type of stimuli (Johansson, Perception & Psychophysics, 1973), only point lights positioned on the limbs of an otherwise invisible agent (e.g. human) are presented, which, despite this highly reduced spatial and temporal information, can provide a strong perception of an acting agent. The objects of the goal-directed actions were not rendered. The responses of the neurons to these dynamic action stimuli were compared to responses to static images of individual snapshots, evenly sampling the action sequence. In addition, we could reduce the stimuli by systematically removing the lower limbs, trunk, etc of the actor to determine which part of the body configuration is necessary to drive the neuron. Other control tests included a receptive field mapping using the most effective stimuli and a reversal test in which the action sequence was played in reverse.

Thus far we have recorded the responses of single temporal neurons in two monkeys exploring a wide range of posterior-anterior positions (PIT-AIT) and dorsal/ventral locations (upper and lower bank STS and lateral convexity). Preliminary results indicate responses to the dynamic images in all regions explored. However, responses selective to particular actions (e.g. lift versus throw) tend to be more prevalent in the anterior than the posterior STS. An example of such a neuron that responded selectively to the action stimuli is shown in Figure 1; note its systematic tuning in the triangular action space. Inspection of the Peri-Stimulus Time Histograms (PSTHs) of the responses showed typically that the neurons responded strongly during particular moments of the action sequence (see also Figure 1). Further analysis using reverse correlation showed that in many selective neurons the responses were the strongest after a particular snapshot. Comparison of the responses to the action stimuli with those to static presentation of the snapshots suggested two sorts of neurons: those responding equally well to static snapshots and the dynamic images and others responding not or much less to the static snapshots (Figure 2). Importantly, these two sorts of neurons provide direct evidence for the existence of "snapshot" and "snapshot motion" units postulated by Giese and Poggio. Most of the neurons responded as well to the motion of the isolated arm as to the whole body configuration.

Thus far, we have no convincing evidence of neurons that responded selectively to the whole action sequence, but this could be due to the sparse sampling of temporal cortex (i.e. missing the right region). Therefore, we are currently planning a monkey fMRI study to find a region that is more strongly activated by the action stimuli than for scrambled action displays, followed by recordings in that region. The latter study follows the spirit of a recent study by Tsao and colleagues (Society for Neuroscience Abstracts, 2005) in which it was reported that >95% of single neurons were selective for faces in a patch of cortex that was defined by fMRI. Thus fMRI could be useful to guide single cell recordings.

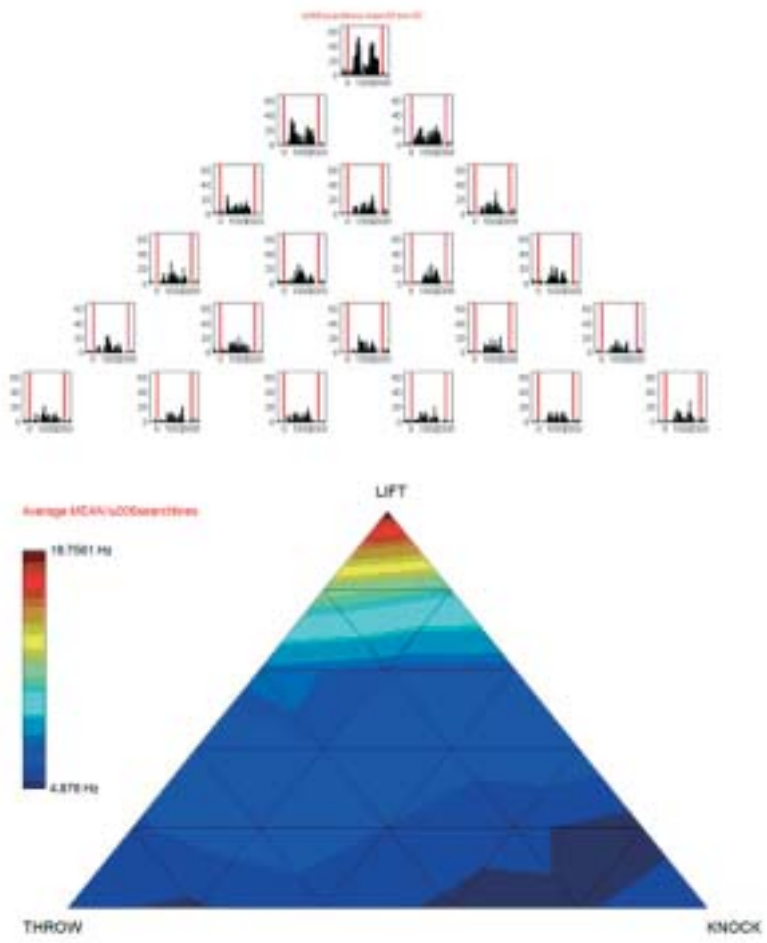

Figure 1. Responses of a single STS neuron to 21 actions of a parameterized action space. These actions consisted of the 3 prototypes (Knock, Throw and Lift actions) and their blends. The responses to the actions are represented in a triangular configuration with the corners corresponding to the prototypes. The stimulus duration was 2000 msecs. The responses are shown as PSTHs (upper panel) and as the mean firing rate (colour coded), averaged across the entire stimulus duration (lower panel). This neuron responded selectively to the Lift action and blended actions containing a strong Lift component. Note the bimodal response profile in the PSTHs indicating that this neuron responded in particular moments of the action sequence.

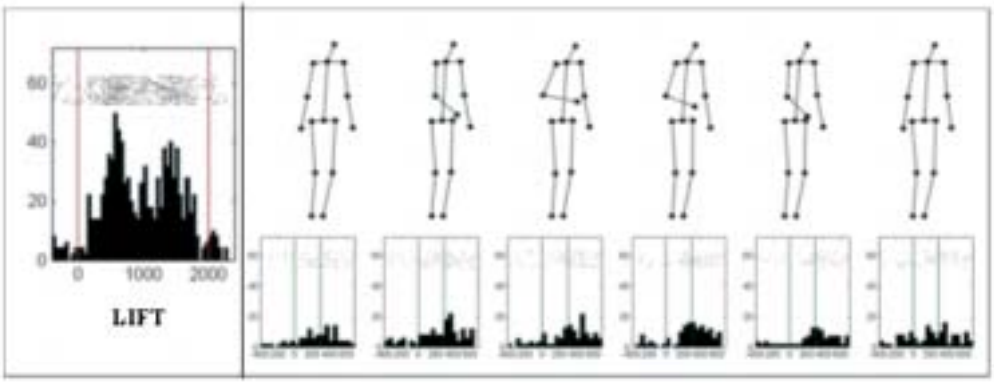

Figure 2. Responses of a single STS neuron to the Lift action sequence (left PSTH and rasterplot) and six static snapshots (right panels). The actual snapshot images are shown above each PSTH. Note that this neuron responded much stronger for the dynamic image sequence (LIFT action) than for the static snapshots (300 msecs stimulus duration). The dynamic action and static snapshot images were presented interleaved in the same test.

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