Geneeskundige Stichting Koningin Elisabeth

Fondation Médicale Reine Elisabeth

verslag rapport

2007

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Geneeskundige Stichting Koningin Elisabeth

2007

Inleiding Verslag Activiteiten van de GSKE – FMRE

In 2007 eindigt de driejaarlijkse kredietperiode (2005-2006-2007) waarin 17 onderzoekteams door de Stichting werden gesubsidieerd. Een samenvattend gedetailleerd verslag is in dit rapport te vinden. Het laat de lezer toe om zich een voortreffelijk beeld te vormen van de kwaliteit van het wetenschappelijke werk van de onderzoekers die door de GSKE worden ondersteund.

Één van de leden van het wetenschappelijk comité, professor Guy Orban werd gevraagd om een aantal lezingen te geven aan het ''College de France" in Parijs. Ter gelegenheid van zijn inaugurele les op 22 maart laatstleden, was Prinses Astrid aanwezig op deze prestigieuze gebeurtenis en vertegenwoordigde Zij er de Stichting.

Eind maart, werd aan het KCE het eindverslag overhandigd betreffende "de zorgbehoeften bij personen met een niet aangeboren hersenletsel " Deze studie, bevolen door KCE en verwezenlijkt in samenwerking met GSKE, heeft het mogelijk gemaakt om concrete voorstellen aan de bevoegde instanties van het land, op het gebied van de gezondheidszorg, in te dienen.

Dat wat in het begin een nieuwigheid was en nadien een traditie is geworden, namelijk de jaarlijkse bezoeken van onze Erevoorzitter Prinses Astrid aan de verschillende laboratoria die door de Stichting worden ondersteund, werd verder gezet. In het voorjaar bracht de Prinses een bezoek aan de laboratoria van professor Yvette Michotte van de VUB en in de herfst aan de laboratoria van professoren Vincent Timmmerman en Peter De Jonghe van de UA .

De jaarlijkse uitreiking van de prijzen van de Stichting vond plaats in het Koninklijk Paleis op 22 mei laatstleden in aanwezigheid van een brede waaier van prominenten van ons land, uit de diplomatieke, culturele en wetenschappelijke wereld. De "Solvay Prize" werd overhandigd aan de professoren Vincent Timmerman en Peter De Jonghe voor hun werkzaamheden betreffende de ziekte van Charcot. De prijs "Baron Van Gysel van Meise" was voor professor Marc Parmentier voor zijn werkzaamheden betreffende specifieke ontvangers. De prijs "Monique Brauns" werd overhandigd aan professor Pierre Maquet voor zijn studies over het geheugen en de slaap.

De zitting werd ingeleid met een opmerkelijke redevoering uitgesproken door Prinses Astrid waarvan de tekst kan geraadpleegd worden op de website van de monarchie.

In de zomer werd de oproep gelanceerd voor nieuwe projecten in het kader van de nieuwe driejaarlijkse wetenschappelijke kredieten voor onderzoek. Niet minder dan 69 projecten zijn voorgelegd voor beoordeling door de leden van het wetenschappelijke comité. Het comité werd uitgebreid met twee buitenlandse deskundigen, professor Kennedy en Rossier van Frankrijk. Het resultaat van het beoordelingswerk van de projecten werd aan de Raad van Bestuur voorgelegd op 4 december. De leden hebben de 16 eersten weerhouden namelijk de teams van de professoren Peter Carmeliet (KULeuven), Anfré Goffinet (UCL),

Emmanuel Hermans (UCL), Peter Janssen (KULeuven), Pierre Maquet (ULg), Yvette Michotte (VUB), Gustave Moonen (Ulg), Marc Parmentier (ULB), Laurence Ris (UMH), Serge Schiffman (ULB), Vincent Timmerman (UA), Christine Van Broeckhoven (UA), Frans Van Roy (UGent), Pierre Vanderhaeghen (ULB), Wim Vanduffel (KULeuven) en Rufin Vogels (KULeuven).

De leden van het wetenschappelijk comité hebben er op gewezen dat de grote meerderheid van de projecten van zeer grote kwaliteit was en dat het opmaken van een rangschikking zeer moeilijk was. Dit is een bewijs van het hoge niveau van het onderzoek in de neurowetenschappen in ons land. Onderzoek waarin de Stichting een zeer belangrijke partner is.

Uiteindelijk danken wij onze Erevoorzitter Prinses Astrid voor Haar aanhoudende en welwillende aandacht voor de werkzaamheden van de Stichting, alsook voor Haar voorturende inzet om de Stichting te vertegenwoordigen op het terrein, waar Ze alle personen ondersteunt die werkzaam zijn in de neurowetenschappen.

Ook dank ik, in naam van alle onderzoekers, de leden van de Raad van Bestuur voor hun verhelderende adviezen en hun permanente zorg om de duurzaamheid van de Stichting te garanderen

Prof. Dr. Baron de Barsy
Wetenschappelijk Directeur
Brussel 30 december 2007

Fondation Médicale Reine Elisabeth

2007

Introduction Rapport d'Activités de la FMRE – GSKE

L'année 2007 termine la triennale (2005-2006-2007) au cours de laquelle 17 équipes de recherches ont été subventionnées par la Fondation. Un rapport détaillé synthétique est inclus dans cet ouvrage et permet au lecteur de se faire une excellente idée de la qualité de la production scientifique des chercheurs soutenus par la FMRE.

Un des membres du comité scientifique, le professeur Guy Orban a été appelé à donner une série conférence au ''Collège de France '' à Paris. A l'occasion de la leçon inaugurale le 22 mars dernier, la princesse Astrid a fait le déplacement pour assister à cet événement prestigieux et y représenter la Fondation.

Fin mars, a été remis à la KCE le rapport final concernant les besoins des patients souffrant de maladie neurologique acquise. Cette étude pluricentrique, ordonnée par le KCE et réalisée en collaboration avec la FMRE, a permis de faire des propositions concrètes aux autorités compétentes du pays dans les domaines des soins de santé.

Ce qui au début état une nouveauté est devenue tradition, à savoir les visites annuelles de notre Présidente d'honneur, la Princesse Astrid aux différents laboratoires soutenus par la Fondation. Au printemps, la Princesse a visité les laboratoires du professeur Yvette Michotte de la VUB et en automne ceux des professeurs Vincent Timmmerman et Peter De Jonghe à l'UA.

La séance annuelle de remise des prix de la Fondation a eu lieu au Palais Royal le 22 mai dernier, en présence d'une large assemblée de personnalités éminentes de notre pays, tant dans le monde scientifique que diplomatique et culturel. Le ''Solvay Prize'' été remis aux Professeurs Vincent Timmerman et Peter De Jonghe pour leurs travaux sur la maladie de Charcot, le prix ''Baron van Gysel de Meise'' au professeur Marc Parmentier pour ses travaux concernant des récepteurs spécifiques et le Prix ''Monique Brauns'' au professeur Pierre Maquet pour ses études sur la mémoire et le sommeil. En guise d'introduction à cette séance, la Princesse Astrid a prononcé un discours remarqué dont le texte peut être consulté sur le site de la monarchie.

En été, a eu lieu l'appel à projet pour une nouvelle triennale de subvention à la recherche.

Pas moins de 69 projets ont été soumis à la lecture des membres du comité scientifique élargi par la présence de deux experts extérieurs, le professeur Kennedy et Rossier, de France.

Le résultat du travail de classement des projets a été soumis au conseil d'administration le

4 décembre, qui a pu retenir les 16 premiers, soit les équipes des professeurs

Peter Carmeliet (KULeuven), Anfré Goffinet (UCL), Emmanuel Hermans (UCL), Peter Janssen (KULeuven), Pierre Maquet (ULg), Yvette Michotte (VUB), Gustave Moonen (Ulg), Marc Parmentier (ULB), Laurence Ris (UMH), Serge Schiffman (ULB), Vincent Timmerman (UA), Christine Van Broeckhoven (UA), Frans Van Roy (UGent), Pierre Vanderhaeghen (ULB), Wim Vanduffel (KULeuven) en Rufin Vogels (KULeuven).

Le comité scientifique a insisté sur le fait que la grande majorité des projets étaient de très grande qualité et que le classement a été rendu très difficile, de ce fait. Ceci témoigne bien du haut niveau de la recherche en neurosciences dans notre pays, recherche dans laquelle la Fondation est un partenaire très important.

Enfin, nous tenons à exprimer tous nos remerciements à notre Présidente d'honneur, la Princesse Astrid, pour son attention soutenue et bienveillante aux travaux de la Fondation, pour son investissement sans compter pour représenter la Fondation sur le terrain et soutenir ainsi toutes les personnes qui travaillent dans ce domaine.

Aussi, que le conseil d'administration pour ses avis éclairés et son soucis permanent de garantir la pérennité de l'institution, soit, au nom de tous les chercheurs vivement, remercié.

Prof. Dr. Baron de Barsy
Directeur Scientifique
Bruxelles 30 décembre 2007

Geneeskundige Stichting Koningin Elisabeth

Activiteiten

2005

12 mei 2005: beursuitreiking aan 17 universitaire onderzoeksploegen

8 december 2005: bezoek van H.K.H. Prinses Astrid aan de laboratoria (Neuro- en Psychofysiologie) van prof. dr. G. Orban (K.U.Leuven).

2006

13 juni 2006: uitreiking van: "UCB Award 2006, voor neurowetenschappelijk onderzoek" aan dr. Pierre Vanderhaeghen en de "Baron van Gysel de Meise Prijs 2006" aan prof. dr. Rufin Vogels in het Koninklijk Paleis te Brussel.

19 december 2006: bezoek van H.K.H. Prinses Astrid aan het Cyclotron Onderzoekscentrum (prof dr. P. Maquet) en aan de Neurobiologische Ontwikkelingsunit (dr. S. Belachew) van de Universiteit Luik.

2007

22 maart 2007: HKH Prinses Astrid woont de inaugurele les bij van professor Guy Orban in het 'Collège de France' te Parijs.

29 maart 2007: publicatie KCE rapport 51A - studie 2005-14 (Chronische zorgbehoeften bij personen met een niet aangeboren hersenletsel (NAH) tussen 18 en 65 jaar) in samenwerking met UZ Gent, ziekenhuis Inkendaal en VUB. De studie is beschikbaar op www.kce.fgov.be (rubriek publicaties) onder referentie KCE reports vol. 51A.

2 mei 2007: bezoek van H.K.H. Prinses Astrid aan de Experimentele Onderzoeksgroep farmacologie (EFAR) van prof dr. Yvette Michotte van de Vrije Universiteit Brussel.

22 mei 2007: uitreiking in het Koninklijk Paleis te Brussel van de

- "Solvay Prize" aan prof. dr. Vincent Timmerman (Universiteit Antwerpen).
- "Baron van Gysel de Meise Prijs 2007" aan prof. dr. Marc Parmentier (Université Libre de Bruxelles).
- "Prijs Monique Brauns" aan prof. dr. Pierre Maquet (Université de Liège).

Juli 2007: Studie project "Behoeften en aanbod inzake zorg en huisvesting van patiënten met Huntington en Multiple Sclerose"

- Het in kaart brengen van de noden inzake opvang en het bestuderen van de huidige mogelijkheden en leemtes op dit vlak.
- Op basis van de vergelijking van deze elementen zullen een aantal beleidsaanbevelingen geformuleerd worden.
- Studie in samenwerking met de heer Larmuseau David, gezondheidseconoom.

26 oktober 2007: bezoek van H.K.H. Prinses Astrid aan de Universiteit Antwerpen, VIB-Departement Moleculaire Genetica, Onderzoeksgroep Perifere Neuropathieën bij prof. dr. Vincent Timmerman en prof. dr. Peter De Jonghe.

Fondation Médicale Reine Elisabeth

Activités

2005

12 mai 2005 : remise des bourses à 17 équipes universitaires de recherche

8 décembre 2005 : la visite de S.A.R. la Princesse Astrid aux laboratoires (Neuro- et Psychofysiologie) du prof. dr. G. Orban (K.U.Leuven).

2006

13 juin 2006 : remise des prix : "UCB Award 2006, pour la recherche en neurosciences" au dr. Pierre Vanderhaeghen et du "Prix Baron van Gysel de Meise" au prof. dr. Rufin Vogels au Palais Royal de Bruxelles.

19 décembre 2006 : La visite de S.A.R la Princesse Astrid au Centre de Recherches Cyclotron (prof P. Maquet) et au Centre de Développement Neurologique (dr. S. Belachew) de l'Université de Liège.

2007

22 mars 2007 : S.A.R. la Princesse Astrid est présente à la leçon inaugurale du Professeur Guy Orban au Collège de France à Paris

29 mars 2007 : publication rapport KCE 51B - étude 2005-14 (Besoin de soins chroniques des personnes âgées de 18 à 65 ans et atteintes de lésions cérébrales acquises) en collaboration avec UZ Gent, ziekenhuis Inkendaal et la VUB. L'étude est disponible au www.centredexpertise.fgov.be (rubrique publication) sous la référence KCE reports vol. 51B.

2 mai 2007 : la visite de S.A.R la Princess Astrid au Groupe de Recherche Pharmacologie Expérimentale (EFAR) du prof. dr. Yvette Michotte de la Vrije Universiteit Brussel.

22 mai 2007 : Remise au Palais Royal de Bruxelles des

- prix "Solvay Prize" au prof. dr. Vincent Timmerman (Universiteit Anwerpen).
- "Prix Baron van Gysel de Meise 2007" au prof. dr. Marc Parmentier (Université Libre de Bruxelles).
- "Prix Monique Brauns" au prof. dr. Pierre Maquet (Université de Liège).

Juillet 2007 : L'étude de projet "Les besoins et l'offre en matière des soins et du logement des patients souffrant de Sclérose en plaques ou la maladie de Huntington."

- Répertorier les besoins en matière d'accueil et examiner les possibilités et les lacunes dans ce domaine.
- Sur base de la comparaison de ces éléments, un nombre de recommandations sera formulé aux décideurs politiques.
- L'étude se fait en collaboration avec Monsieur David Larmuseau, économiste en santé.

26 octobre 2007 : la visite de S.A.R la Princesse Astrid à l'Université d'Anvers, VIB - Département Génétic Moléculaire, Groupe de Recherche des Neuropathies périmétriques chez le prof. dr. Vincent Timmerman et le prof. dr. Peter De Jonghe.

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-HT7 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/AT4 receptor system in learning and memory processes.

Final Reports of the University Research Groups, supported by the Queen Elisabeth Medical Fondation in collaboration with the following Professors and Doctors (2007)

Final Report of the Research Group of

Dr. Belachew S.

Université de Liège

(U.Lg)

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Unraveling the role of Cyclin-Dependent Kinase 2 during postnatal gliogenesis and neurogenesis.

A. Aims

Improving our understanding of the intrinsic regulatory mechanisms that control proliferation of neuronal and glial progenitors in the adult CNS will be crucial for designing new strategies of treatment for brain diseases. In demyelinating disorders, remyelination notably fails because of a defective recruitment of adult oligodendrocyte progenitor cells (OPC). Likewise, adult neurogenesis may also be limited by the quiescent state of neural progenitor cells (NPC) located in the neurogenic zones of the brain, including the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles.

Cyclin-Dependent Kinase 2 (Cdk2), which controls G1/S transition in eukaryotic cell cycle, was recently shown to be dispensable during embryonic deve lopment, since Cdk2-null mice develop normally until adulthood (Berthet et al., 2003). Cdk2 was previously shown to be downregulated in adult OPCs *in vitro* (Belachew et al., 2002) and some cell cycle regulators connected to Cdk2, including $p27^{kip1}$ (Doetsch et al., 2002), $p21^{cip1}$ (Kippin et al, 2005) or E2F1 (Cooper-Kuhn CM et al., 2002), specifically regulate neurogenesis in the adult brain.

The present work was meant to address the specific requirement for Cdk2 *in vivo*:

1) During proliferation events which persist through the entire life in the DG and the SVZ (paragraph 1 and 3)

2) During white matter development in the normal uninjured brain and in a model of acquired demyelination (paragraph 2)

B. Studies and Results

1. Cdk2 is critical for proliferation and self-renewal of neural progenitor cells in the adult subventricular zone.

We investigated the function of cyclin-dependent kinase 2 (Cdk2) in neural progenitor cells during postnatal development. Chondroitin sulfate proteoglycan (NG2)-expressing progenitor cells of the subventricular zone (SVZ) show no significant difference in density and proliferation between Cdk2 (-/-) and wild-type mice at perinatal ages and are reduced only in adult Cdk2(-/-) mice (Figure 1).

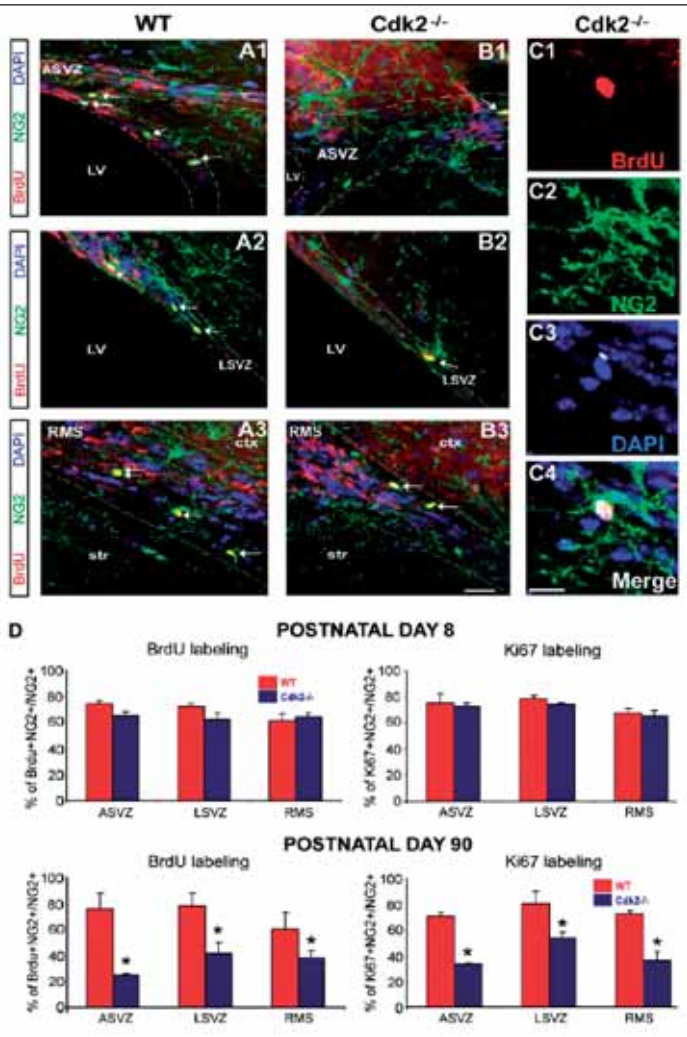

Figure 1. Decreased proliferation of NG2-expressing progenitors in neurogenic areas of the adult Cdk2−/− mouse. (A–C) Tricolored images show NG2+ progenitor cells (green) in the ASVZ, LSVZ, and RMS (LV, lateral ventricle; ctx, cortex; str, striatum) on sagittal sections obtained from wild-type (A1–3) and Cdk2−/− (B1–3) mice stained with anti-BrdU (red) and DAPI (blue). Arrows indicate proliferating NG2+ progenitors. White dotted lines delineate the ASVZ (A1 and B1), LSVZ (A2 and B2), and RMS (A3 and B3). (C1–4) Cdk2−/− mouse. Magnifi ed view of NG2+ progenitors (C2, anti-NG2) labeled with anti-BrdU (C1) and DAPI (C3). Merged image is shown in C4. (D) Percentages of double-labeled cells. At P8, no differences in the percentage of NG2+–BrdU+ or NG2+–Ki67+ cells were observed in the ASVZ, LSVZ, and RMS between wild-type and Cdk2−/− mice (D, top left and right), whereas at P90 there was a signifi cant reduction in the percentage of proliferating NG2+ cells in the Cdk2−/− brain (D, bottom left and right). Results are expressed as means ± the SEM. *, $P < 0.05$; results were analyzed by a t test (six to eight hemispheres were taken for analysis for each age and marker). Bars: (A and B) 50 µm; (C) 12 µm.

Adult Cdk2(-/-) SVZ cells in culture display decreased self-renewal capacity and enhanced differentiation. Compensatory mechanisms in perinatal Cdk2(-/-) SVZ cells, which persist until postnatal day 15, involve increased Cdk4 expression that results in retinoblastoma protein inactivation. A subsequent decline in Cdk4 activity to wild-type levels in postnatal day 28 Cdk2(-/-) cells coincides with lower NG2+ proliferation and self-renewal capacity similar to adult levels (Figure 2).

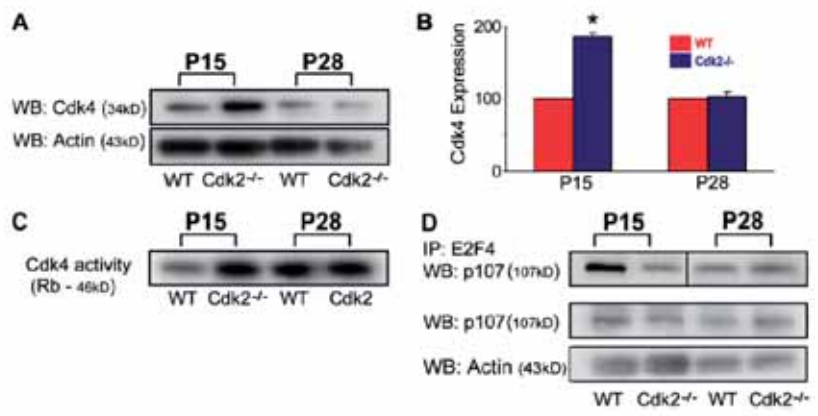

Figure 2. Up-regulation of Cdk4 expression and activity in the Cdk2−/− SVZ decline to adult levels between P15 and 28. (A and B) SVZ tissue lysates were immunoblotted with anti-Cdk4 antibodies and band intensities were measured and normalized to actin. Note that the increase inCdk4 expression in Cdk2−/− tissue is developmentally regulated and was lost at P28. Each histogram was obtained from an independent Western blot analysis of three to four SVZs. *, P <0.05; results were analyzed by a t test. (C) Cdk4 activity was up-regulated in Cdk2−/− SVZ at P15 but not P28. Cdk4 activity was measured using glutathione S-transferase Rb protein as a substrate. (D) At P15, E2F4-bound p107 was lower in Cdk2−/− than in wild-type SVZ, which indicates enhanced activation of the Cdk4 pathway. Conversely, at P28, E2F4-bound p107was similar in Cdk2−/− and wild-type SVZ. E2F4-bound p107 was coimmunoprecipitated withanti-E2F4 antibodies and probed on Western blot with anti-p107 antibodies. Importantly,p107expression levels did not change at either rage in Cdk2−/− versus wild-type SVZ as shown by Western blot with anti-p107 antibodies.

Cdk4 silencing in perinatal Cdk2(-/-) SVZ cells abolishes Cdk4 up-regulation and reduces cell proliferation and self- renewal to adult levels. Conversely, Cdk4 overexpression in adult SVZ cells restores proliferative capacity to wild-type levels (Figure 3).

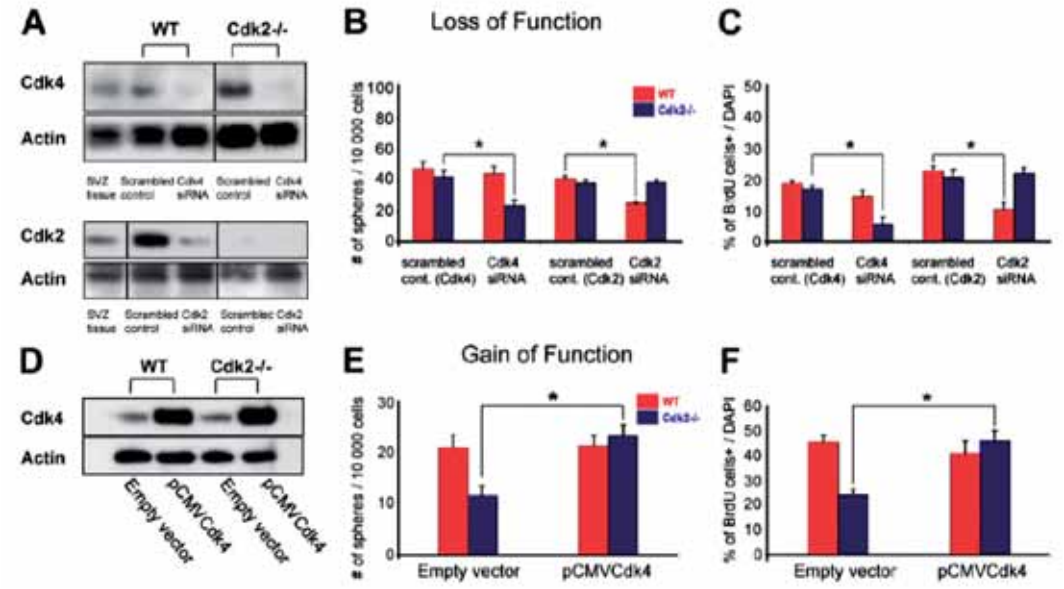

Figure 3. siRNA-induced knockdown of Cdk4 and 2 inhibits proliferation in P8 Cdk2−/− and wild-type SVZ cell cultures, respectively. (A–C) Loss of-function experiments in perinatal SVZ cells. Cells from P8 brains were transfected with scrambled (control), Cdk4-silencing, or Cdk2-silencing siRNA and harvested 24 h after transfection. (A) Cell and SVZ tissue lysates were immunoblotted with anti-Cdk4 and -Cdk2 antibodies and band intensities from siRNA-treated cells were measured and normalized to actin. In both wild-type and Cdk2−/− cells, a reduction of 50–70% in Cdk4 levels was obtained after siRNA transfection. Wild-type P8 SVZ tissue lysate was also used as a positive control for Cdk4 expression. Transfection with Cdk2 siRNA decreased Cdk2 expression in wild-type SVZ cells by 50% but did not modify Cdk2 expression in Cdk2−/− P8 SVZ cells. Wild-type P8 SVZ tissue lysate was also used as a positive control for Cdk2 expression. Black lines indicate that the intervening lanes have been spliced out. (B) Transfection of Cdk2−/− cells with scrambled siRNA control did not modify neurosphere formation but Cdk4 siRNA

caused a signifi cant decrease in the number of neurospheres. No effect was observed in wild-type cultures. Transfection with Cdk2 siRNA reduced the number of neurospheres in wild-type cultures as compared with treatment with scrambled control. No effect was observed in Cdk2−/− cells. Data were obtained from second passage neurospheres of three independent experiments. (C) BrdU immunolabeling showed that neural progenitor cell proliferation was impaired in Cdk2−/− cultures after Cdk4 siRNA treatment but not in wild-type cultures. After Cdk2 siRNA transfection, proliferation was impaired in wild-type but not Cdk2−/− cultures. Data represent BrdU+ cells as percentages of total DAPI-labeled cells and were obtained from three separate experiments (three independent cell cultures). The total number of cells counted was 454 (scrambled control Cdk4), 782 (scrambled control Cdk2), 458 (Cdk4 siRNA), and 636 (Cdk2 siRNA) for the wild type; and 647 (scrambled control Cdk4), 765 (scrambled control Cdk2), 624 (Cdk4 siRNA), and 643 (Cdk2 siRNA) for Cdk2−/−. (D-F) Gain-of-function experiments in adult SVZ cells. Plasmid pCMVCdk4 and empty vector were transfected to P90 wild-type and Cdk2−/− cells. (D) Western blot analysis shows higher Cdk4 expression in both wild-type and Cdk2−/− SVZ cells after transfection with pCMV-Cdk4 as compared with transfection with an empty vector. (E) Transfection of Cdk2−/− cells withpCMV-Cdk4 caused a signifi cant increase in the number of neurospheres compared with wild-type cells. Data were obtained from second passage neurospheres of three independent experiments. (F) BrdU incorporation assays. Cdk4 overexpression did not modify cell proliferation in wild-type cells but greatly increased cell proliferation in Cdk2−/− cells to levels similar to the wild type. Data were obtained from three independent experiments. The total number of cells counted was 511 and 451 for the empty vector (wild type and Cdk2−/−, respectively) and 425 and 608 for pCMV-Cdk4 (wild type and Cdk2−/−, respectively). Data are expressed as means ± the SEM. *, P < 0.05; results were analyzed by a t-test.

Thus, although Cdk2 is functionally redundant in perinatal SVZ, it is important for adult progenitor cell proliferation and self-renewal through age-dependent regulation of Cdk4.

2. Differential requirement of Cdk2 for normal white matter development and myelin repair

To challenge the role of Cdk2 in oligodendrocyte development, 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) (Figure 4).

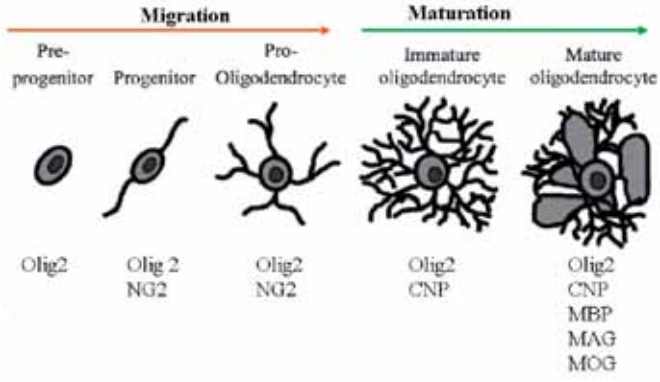

Figure 4: Specific markers of oligodendrocyte differentiation.

The density and distribution of CNPase-, MAG- and Olig2-expressing cells observed was identical in the corpus callosum and sub-cortical white matter of wild-type (WT) versus Cdk2 -/- adult mice (3-months old) (Figure 5).

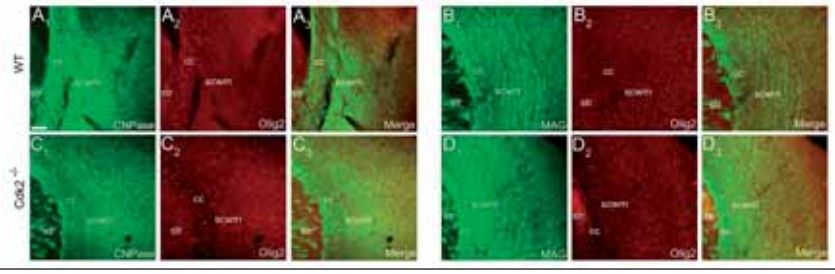

***Figure 5** : Single plane confocal images of 50 µm-thick sections of subcortical white matter (scwm), and corpus callosum (cc) areas from P90 wild-type (WT) and Cdk2-deficient mice (Cdk2 -/-) immunostained for CNPase/Olig2 (left pannels) or MAG/Olig2 (right pannels) in order to show that the density and distribution of these oligodendroglial lineage markers were similar between WT and Cdk2 -/- mice. Scale bar = 100 µm.*

Similar results were obtained from the cerebellar white matter and striatum (data not shown). Finally, the absolute density of Olig2-expressing cells (i.e. the entire oligodendroglial lineage cells) in the corpus callosum and of NG2-expressing OPCs in the corpus callosum was not different between WT and Cdk2-/- adult mice (Figure 6).

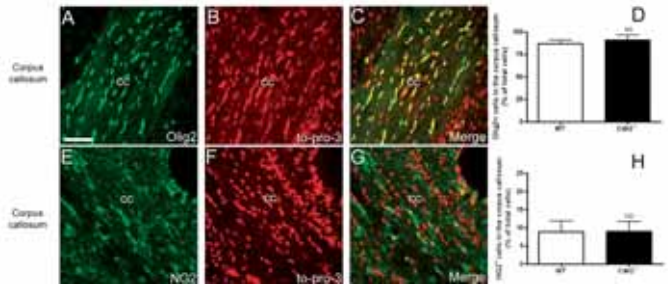

Figure 6: (A→C, E→G) Single plane confocal images of the corpus callosum (cc) area from P90 wild-type (WT) and Cdk2-deficient mice (Cdk2 -/-) Images were obtained from 50 µm-thick coronal sections immunostained for Olig2 or NG2 (white arrows point towards NG2+ cells). TO-PRO®-3 was used for nuclear counterstaining. Scale bar = 50 µm. (D, H) Histograms representing the overall density of Olig2+ or NG2+ cells in the corpus callosum (% of total cells) (mean ± SD, Student's t-test, NS= not significant).

These data provide evidence that Cdk2 does not appear to be essential for normal developmental myelination.

We next used, as previously described (Nait-Oumesmar et al. 1999; Decker et al. 2002), a model of focal lysolecithin-induced lesion of the corpus callosum in order to challenge the role of Cdk2 in OPC proliferation and oligodendrogenesis following acquired non-autoimmune demyelination. Ten animals of each genotype (wt and cdk2-/- mice) deeply anesthetized with isofluran were positioned in a stereotaxic frame and injected unilaterally into the corpus callosum, using appropriate coordinates (1.5 mm anterior to the bregma, 1 mm lateral, and 1.8 mm deep from the skull surface) with a 10µl Hamilton syringe. Five animals of each genotype were injected with 2µl of a 1% lysolecithin solution (LPC, Sigma) in 0.9% NaCl with a flow rate of 0.5µl/min (Figure 7). Control animals (5) were injected with 2µl of saline solution only. Animals were perfused 4 days after stereotaxic injection. To compare proliferation in response to demyelination between WT and Cdk2-/- mice, 12 µm tissue sections were processed for MBP and Ki67. Previous studies revealed that the percentage of Ki67+ cells in adult SVZ was lower in the Cdk2-/- mouse than in WT mouse (cf. first paragraph). Our previous data also showed that demyelination enhances proliferation in the SVZ (Nait-Oumesmar et al. 1999; Picard-Riera et al. 2002). We therefore questioned whether this increase of cell proliferation in response to demyelination is influenced by the loss of Cdk2. This was indeed the case since in the SVZ of demyelinated animals, we observed a 2 fold increase of Ki67+ cells in WT mice compared to Cdk2-/- mice (Figure 8).

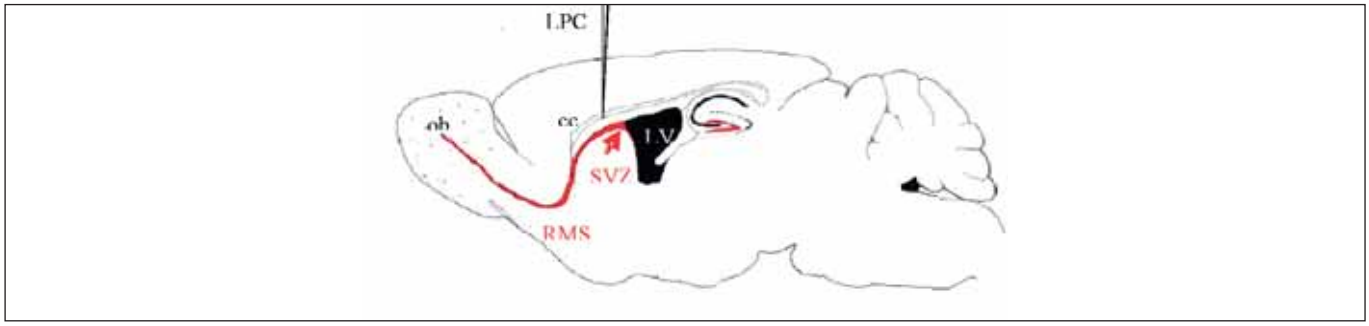

Figure 7: Localization of the injection point in the corpus callosum (cc), above the rostral migratory stream (RMS) in which cells arising from the subventricular zone (SVZ) migrate to the olfactory bulb (ob). LV: lateral ventricle.

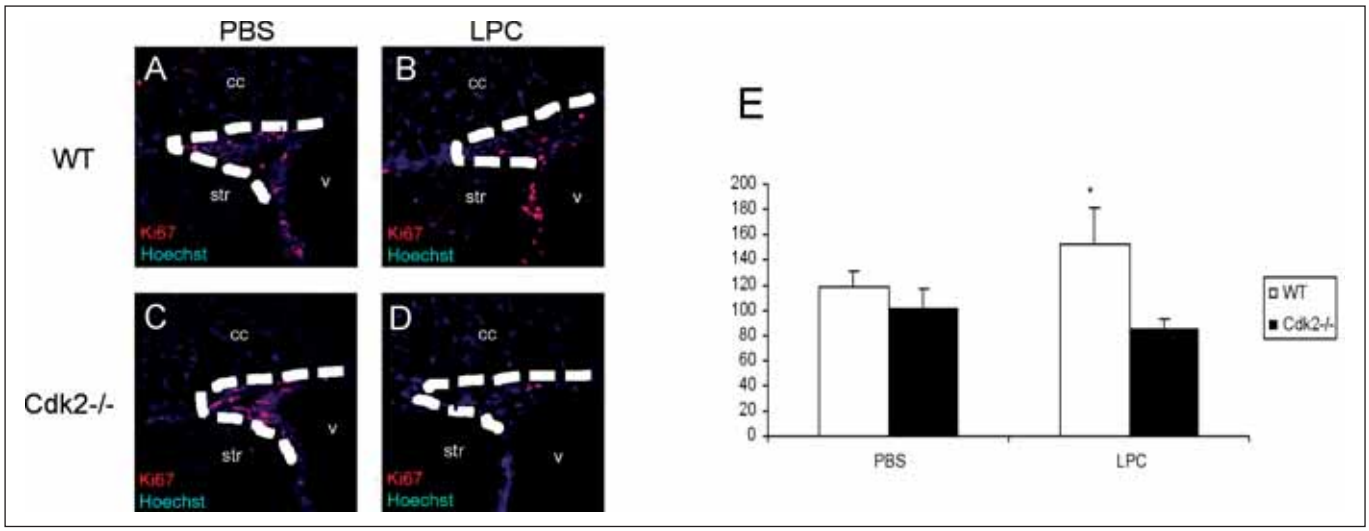

Figure 8: (AgD) Proliferation in adult SVZ of normal (A, C) and demyelinated animals (B, D). Comparison between wild-type (A, B) and Cdk2 -/- animals (C, D) with Ki67 labelling. Scale bar = 50 µm. cc: corpus callosum ; str : striatum ; v : ventricle. (E) Histogram representing the number of SVZ proliferating cells of WT and Cdk2 -/- animals in each conditions (mean ± SD, Student's t-test, * = P<0.05).

In order to characterize the proliferating cells affected by the loss of Cdk2, we performed double immunolabelling for the transcription factor Olig2 and Ki67. A significant decrease in the percentage of Olig2/Ki67+ cells was observed in the SVZ of Cdk2-/- demyelinated animals (Figure 9).

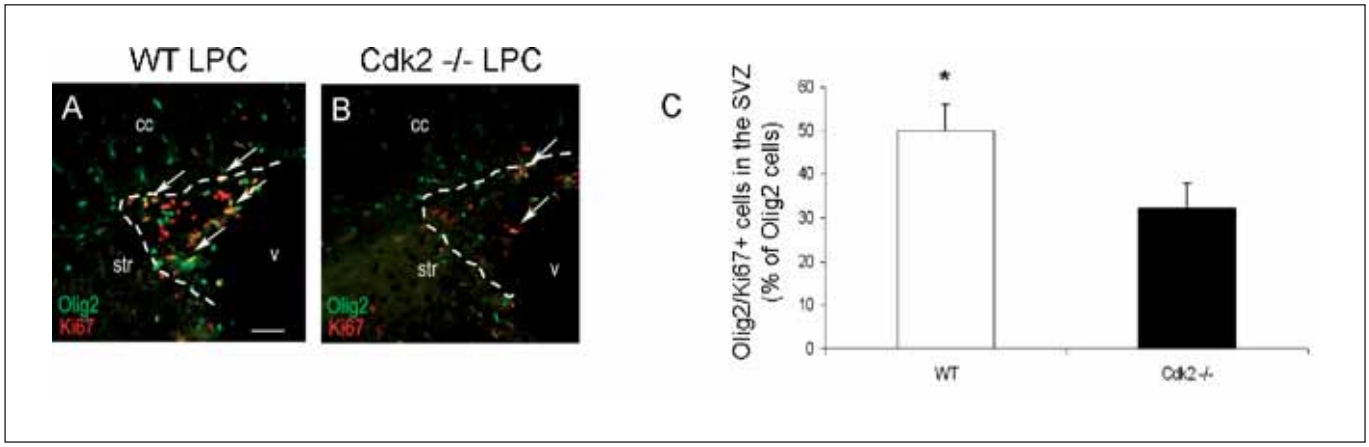

Figure 9: (A,B) Olig2 proliferating cells in the SVZ of demyelinated animals. Comparison between wild-type (A) and Cdk2 -/- animals (B) with Ki67 labelling. Scale bar = 50 µm. cc: corpus callosum ; str : striatum ; v : ventricle. (C) Histogram representing the percentage of Olig2 cells which are proliferating in the SVZ (mean ± SD, Student's t-test, * = P<0.05).

There was no difference in the number of SVZ Olig2/Ki67 cells between mutant and wild-type mice when injected with PBS (data not shown).

Demyelination is also known to trigger proliferation of glial cells in the lesion area. We found that the number of Ki67+ cells increased in WT mice compared to Cdk2-/- mutants (Figure 10).

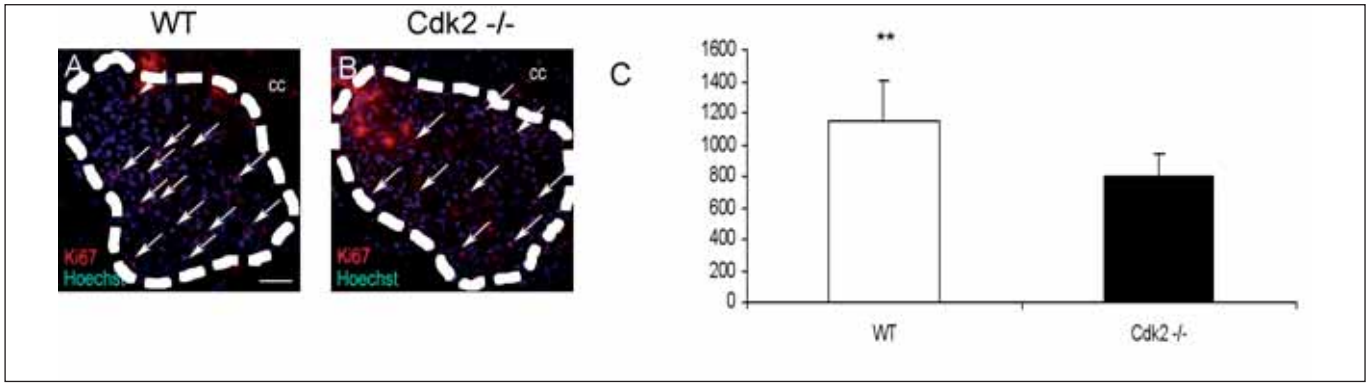

Figure 10: Proliferation in a focal lesion of demyelination induced by LPC in the corpus callosum (cc). Comparison between wild-type (A) and Cdk2 -/- animals (B) with Ki67 labelling. Scale bar = 50 µm. (C) Histogram representing the number of proliferating cells per mm2 of demyelinating lesion in WT and Cdk2 -/- animals (mean ± SD, Student's t-test, ** = P<0.001).

To investigate whether OPCs proliferate, we performed double labelling for Olig2 and Ki67. We found that the number of Olig2+/Ki67+ cells was reduced in lesions of Cdk2 -/- mice thus suggesting that oligodendrocytes contribute but are not the only cell type involved in the reduction of cell proliferation which occurs in the lesion (Figure 11).

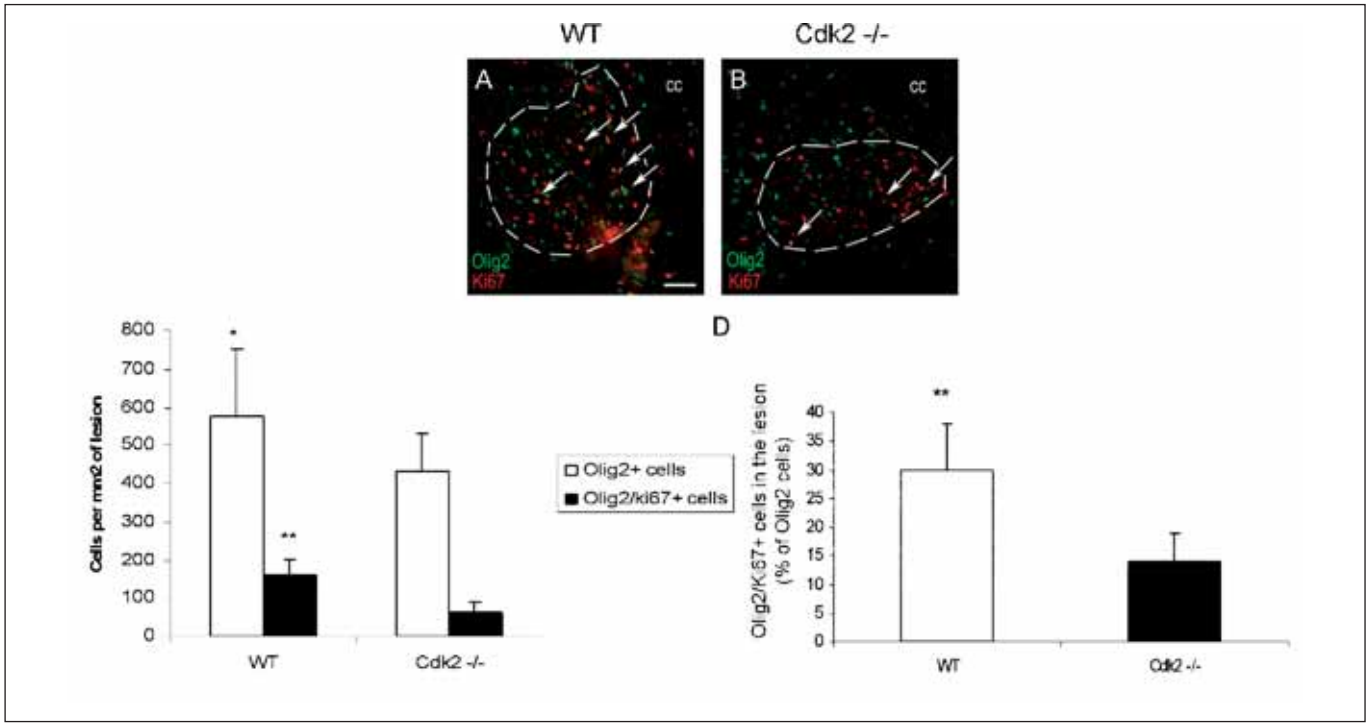

Figure 11: (A,B) Olig2 proliferating cells in demyelinated lesions of the corpus callosum (cc). Comparison between wild-type (A) and Cdk2 -/- animals (B) with Ki67 labelling. Scale bar = 50 µm. (C) Histogram representing the number of Olig2+ and Olig2+/Ki67+ cells per mm2 of lesion in WT and Cdk2 -/- animals..(D) Histogram representing the percentage of Olig2 cells which are proliferating in the lesion (mean ± SD, Student's t-test, * = P<0,05, ** = P<0.001).

In conclusion, the preliminary data showed that Cdk2 does not appear to be essential for cell cycle kinetics and thus normal developmental myelination suggesting its function may be compensated in neonatal OPC. However, these recent data by Caillava et al. demontrate that it seems to be involved in cell cycle kinetics in adult neural precursors and OPC following demyelination.

3. Cdk2 is dispensable for adult hippocampal neurogenesis

Granule neurons of the dentate gyrus (DG) of the hippocampus undergo continuous renewal throughout life. Among cell-cycle regulators, cyclin-dependent kinase 2 (Cdk2) is considered a major regulator of S phase entry. We used Cdk2-deficient mice to decipher the involvement of Cdk2 in the generation of new neurons in the adult hippocampus. The quantification of cell cycle markers first revealed that Cdk2 invalidation does not influence spontaneous or seizure-induced proliferation of neural progenitor cells (NPCs) in the adult DG (Figure 12).

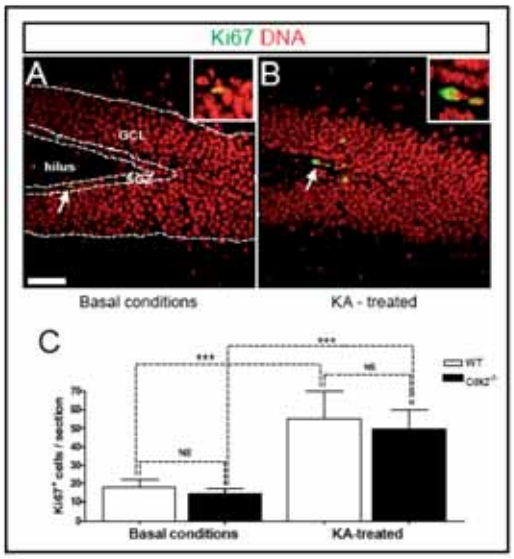

Figure 12. The absence of Cdk2 does not affect the number of proliferating NPCs in the adult dentate gyrus.
(A,B) Representative single-plane confocal images displaying proliferating Ki67+ cells in the DG from adult mice in basal conditions (A) or treated with KA (B). Hoechst 33342 was used for nuclear counterstaining (DNA). Arrows indicate cells which are shown at higher magnification in boxed areas. GCL=granule cell layer and SGZ=subgranular zone.
(C) Histograms representing the number of Ki67+ cells per 40µm-thick section of DG in basal conditions (mean±SD, n=8 animals per genotype) or after KA treatment (mean±SD, n=4 animals per genotype). Scale bar for A and B = 50µm. NS=not significant, ***=P <0.001

Using bromodeoxyuridine incorporation assays, we showed also that the adult production of mature newborn granule neurons was identical in wild-type (WT) and Cdk2-deficient mice (Figure 13).

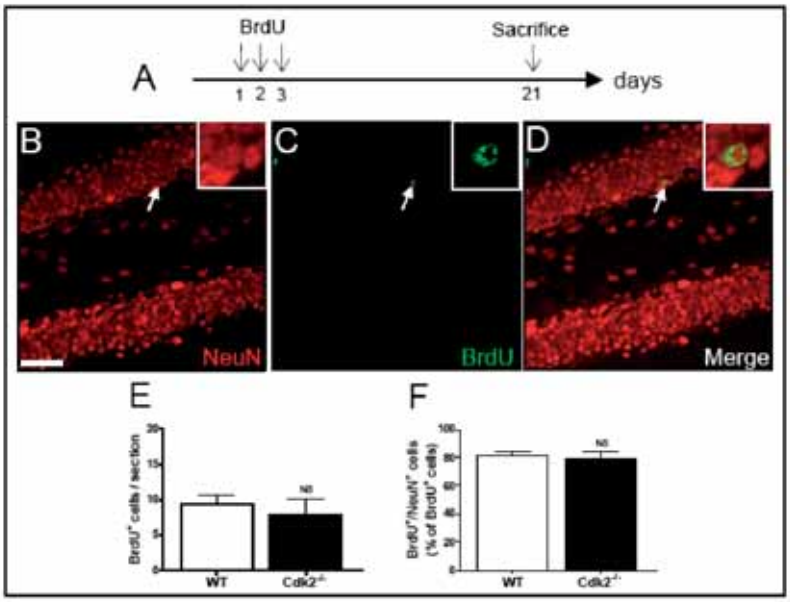

Figure 13. The lack of Cdk2 does not impair the generation of new neurons in the adult dentate gyrus.
(A) Adult WT and Cdk2-/- mice were daily injected with BrdU during three consecutive days and animals were sacrificed 3 weeks later. (B-D) Representative single-plane confocal images displaying an adult newly-generated DG neuron labelled with antibodies directed against the neuronal post-mitotic marker NeuN (B) and BrdU (C). Arrows indicate the cells which are shown at higher magnification in boxed areas. (E, F). Histograms representing (E) the number of newly-generated BrdU+ cells per 40µm-thick section of DG after 3 weeks (mean±SD, n=7 animals per genotype) and (F) the percentage of BrdU+ cells expressing NeuN (mean±□SD, n=7 animals per genotype). Scale bar for B, C and D=50µm. NS=not significant.

Finally, we demonstrated that the number of apoptotic cells was similar between the DG of WT and that of Cdk2-/- mice (Figure 14).

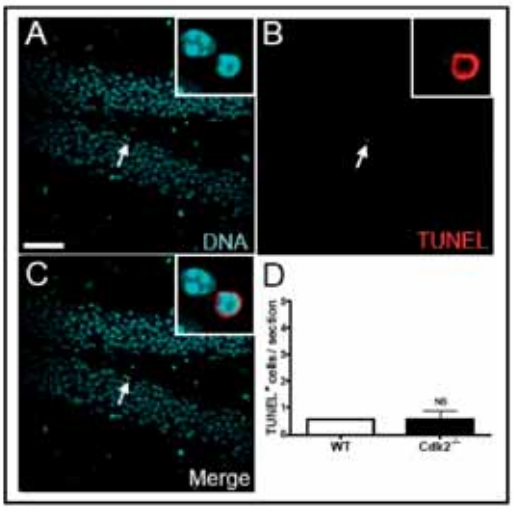

Figure 14. Cdk2 functional deletion does not change the rate of cell death in the adult dentate gyrus
(A-C) Representative single-plane confocal images displaying a TUNEL+ cell in the adult DG. Hoechst 33342 was used for nuclear counterstaining. Arrows indicate the cells which are shown at higher magnification in boxed areas. (D) Histograms representing the number of TUNEL+ cells per 40µm-thick section of DG (mean□±SD, n=5 animals per genotype). Scale bar for A, B and C=50□m. NS=not significant.

Our results indicate that Cdk2 is not required for proliferation, differentiation and survival of adult-born dentate granule neurons *in vivo*. These data emphasize that functional redundancies also occur in the adult brain at the level of neural progenitor cell cycle regulation during hippocampal neurogenesis.

C. Publications and Abstracts resulting from this grant

1. Publications

- Nguyen L, Borgs L, Vandenbosch R, Mangin JM, Beukelaers P, Moonen G, Gallo V, Malgrange B, Belachew S: The Yin and Yang of cell cycle progression and differentiation in the oligodendroglial lineage. Ment Retard Dev Disabil Res Rev. 2006;12(2):85-96.
- Vandenbosch R, Borgs L, Beukelaers P, Foidart A, Nguyen L, Moonen G, Berthet C, Kaldis P, Gallo V, Belachew S, Malgrange B: Cdk2 is Dispensable for Adult Hippocampal Neurogenesis. Cell Cycle. 2007 Dec 15; 6(24):3065-3069.
- Jablonska B, Aguirre A, Vandenbosch R, Belachew S, Berthet C, Kaldis P, Gallo V : Cdk2 is critical for proliferation and self-renewal of neural progenitor cells in the adult subventricular zone.J Cell Biol. 2007 Dec 17;179(6):1231-45.

2. Abstracts

- Vandenbosch R, Borgs L, Jablonska B, Malgrange B, Breuskin I, Moonen G, Gallo V, Belachew S. White matter development and adult neurogenesis in cdk2 deficient mice. 9th European Graduate School of Neuroscience (EURON) Ph.D. student Meeting, Université de Liège, Liège, Belgium, September 8-9, 2005.
- Vandenbosch R, Jablonska B, Berthet C, Borgs L, Malgrange B, Breuskin I, Moonen G, Kaldis P, Gallo V, Belachew S. White matter development and adult hippocampal neurogenesis in Cdk2-deficient mice. 35th annual meeting of the Society for Neuroscience, Washington DC, USA, November 12-16, 2005.
- Vandenbosch R, Jablonska B, Berthet C, Borgs L, Malgrange B, Breuskin I, Moonen G, Kaldis P, Gallo V, Belachew S. White matter development and adult hippocampal neurogenesis in Cdk2-deficient mice. ***Autumn Meeting 2005 of the Belgian Society of Cell and Developmental Biology***, Hasselt, Belgium, Saturday October 15, 2005.
- Caillava C, Vandenbosch R, Kaldis P, Berthet C, Gallo V, Malgrange, B, Belachew S and Baron-Van Evercooren A. Exploring the requirement of Cdk2 for normal white matter development and myelin repair following acquired demyelination. ***1st meeting of the European Leukodystrophy Association (ELA),*** Paris, France, October 5-7 2006.
- Vandenbosch R, Caillava C, Kaldis P, Berthet C, Gallo V, Malgrange, B, Baron-Van Evercooren A and Belachew S. Exploring the requirement of Cdk2 for normal white matter development and myelin repair following acquired demyelination. ***Joint Meeting of Belgian Neuroscience Societies (Neurobelgium) 2006***, Lac de Genval, Belgium, November 2006.
- Vandenbosch R, Borgs L, Beukelaers P, Nguyen L, Foidart A, Moonen G, Gallo V, Berthet C, Kaldis P, Malgrange B and Belachew S. Functional effects of Cdk2 deletion on adult hippocampal neurogenesis. ***7th bi-annual meeting of the Belgian Society for Neuroscience***, Antwerp, Belgium, Monday May 7, 2007.
- C. Caillava, R. Vandenbosch, P. Kaldis, C. Berthet, V. Gallo, B. Malgrange, S. Belachew and A. Baron-Van Evercooren, Exploring the requirement of Cdk2 for normal white matter development and myelin repair, 2007, ***Franco-Italian ARSEP meeting***.
- C. Caillava, R. Vandenbosch, P. Kaldis, C. Berthet, V. Gallo, B. Malgrange, S. Belachew and A. Baron-Van Evercooren, Exploring the requirement of Cdk2 for normal white matter development and myelin repair, 2007, ***Euroglia***.
- Vandenbosch R, Borgs L, Beukelaers P, Nguyen L, Foidart A, Moonen G, Gallo V, Berthet C, Kaldis P, Malgrange B and Belachew S. Functional effects of Cdk2 deletion on adult hippocampal neurogenesis. ***37th annual meeting of the Society for Neuroscience***, San Diego, USA, November 3-7, 2007.

Final Report of the Research Group of

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Experimental Analysis of Cerebellar Coding

Introduction

Purkinje cell simple spike trains are the sole result of the computations performed by the cerebellar cortex. The cerebellum plays an important role in motor control and more particular in its dynamics by adapting the relative timing of muscle activation (Ito, 1984). It is also involved in several other tasks requiring the precise representation of temporal information (Ivry and Spencer, 2004). Therefore it is surprising that studies of coding by Purkinje cells have usually considered only simple spike and complex spike mean firing frequencies (Shidara et al., 1993; Kitazawa et al., 1998). Little attention has been paid to the temporal structure of the spike train though spike timing has been shown to encode additional information in many systems (Rieke et al., 1997).

During the past 3 years we have systematically explored the temporal structure of simple spike in the cerebellum and reported several new findings. These are here summarized starting from how they may affect the target neurons in deep cerebellar nuclei (DCN). Subsequently we describe the results in more detail.

DCN neurons are known to produce strong rebound spikes upon release from inhibition. Such release requires a synchronized pause in firing of afferent Purkinje cells, we indeed found that 13% of pauses where synchronized among neighboring Purkinje cells (Shin and De Schutter, 2006). Therefore the pauses may form a temporal code in simple spike trains. If evoking rebound spikes is an important coding principle, Purkinje cells must be able to regulate them. Surprisingly, we found that SS firing contains long stretches of regular firing, mostly at faster rates (Shin et al., 2007b). These regular patterns, together with the known fast depression of Purkinje cell to DCN synapses, form a perfect rate code, where firing rate translates into steady state inhibition of DCN neurons. We propose that this rate code controls the amplitude of rebound spikes that may be evoked shortly afterwards. Another way to regulate rebound spikes is by changing their duration. We found that the proportion of pauses in simple spike trains is highly variable between Purkinje cells, which may be linked to an underlying variable excitability (Achard and De Schutter, 2006). Another cause may be synaptic plasticity. Indeed, we found that induction of long-term depression of the parallel fiber synapse shortens the simple spike pause evoked by strong parallel fiber stimuli (Steuber et al., 2007).

In addition we studied the sensitivity of cerebellar Golgi cells to the temporal structure of activating stimuli.

Dynamic synchronization of Purkinje cell simple spikes

Published as Shin and De Schutter, *Journal of Neurophysiology* (2006)

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 (Ebner and Bloedel, 1981). 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 µm.

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.

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).

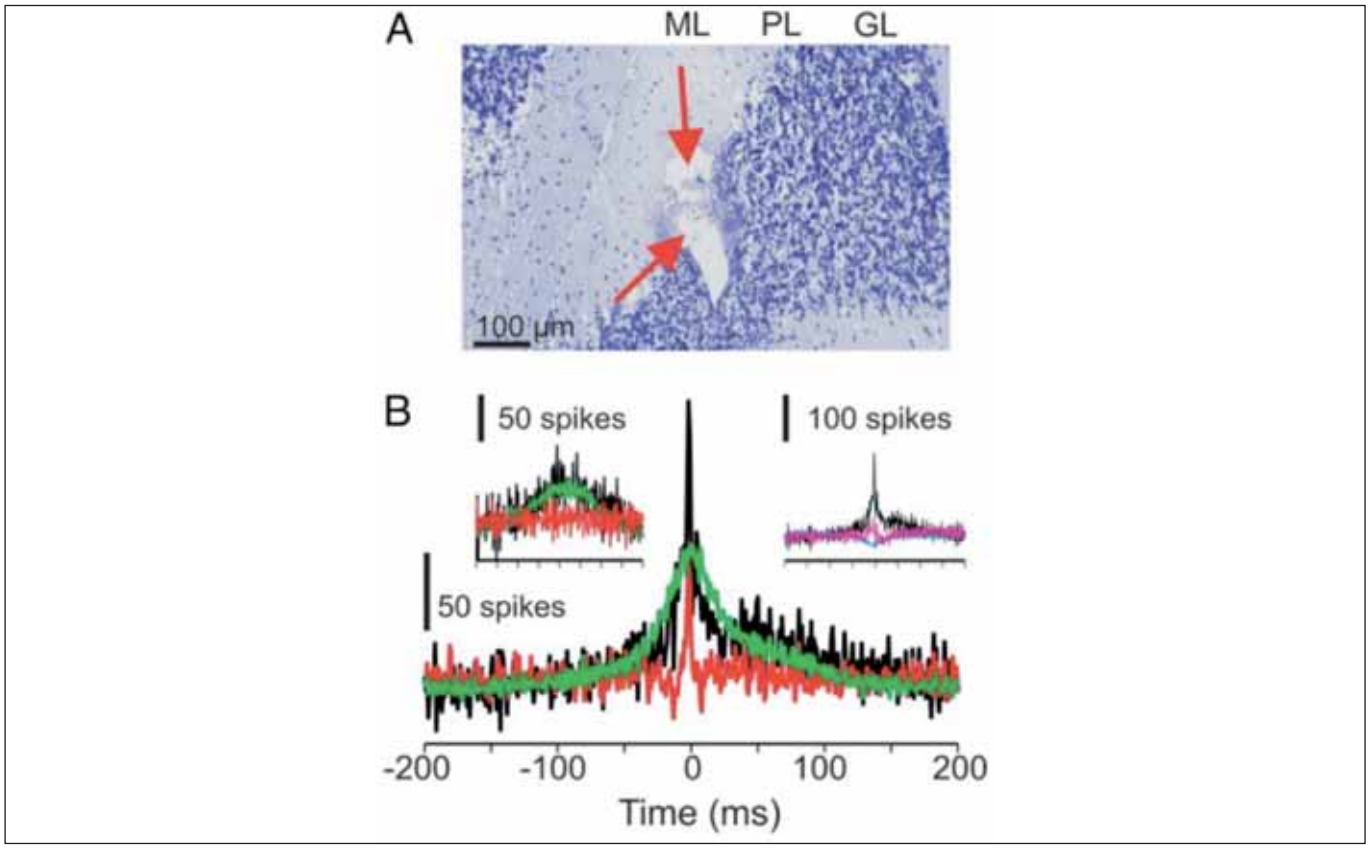

Figure 1: Cross-correlation of nearby Purkinje cell simple spike trains.

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 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, which was not different from single cell recordings ($p > 0.9$, Student's t test). Using this criterion spikes were classified as either pause spikes (spikes beginning, surrounding, or ending a pause) or non-pause spikes. In the 8 PC pairs 73.2 ± 4.1 % of spikes were classified as pause spikes.

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

The preceding analysis presented was based on cross-correlation of specific categories of spikes. This does not guarantee that pauses were highly synchronized because a spike at the start of a pause in one PC can be synchronous with a spike at the end of a pause in the other PC. Therefore in a final analysis, we selected all Pt synchronous within and checked whether the corresponding pauses where also synchronized. Overall we found that 13% of all pauses were synchronized.

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 μm fire 13% 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.

Regular patterns in Purkinje cell simple spike trains

Published as Shin et al., *PLoS One* (2007)

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

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

Therefore it is surprising that studies of coding by PCs have usually considered only SS (Shidara et al., 1993; Coltz et al., 1999; Kahlon and Lisberger, 2000) and CS (Kitazawa et al., 1998; Goossens et al., 2004) mean firing frequencies. Little attention has been paid to the temporal structure of the spike train though spike timing has been shown to encode additional information in many systems (Rieke et al., 1997; Vanrullen et al., 2005).

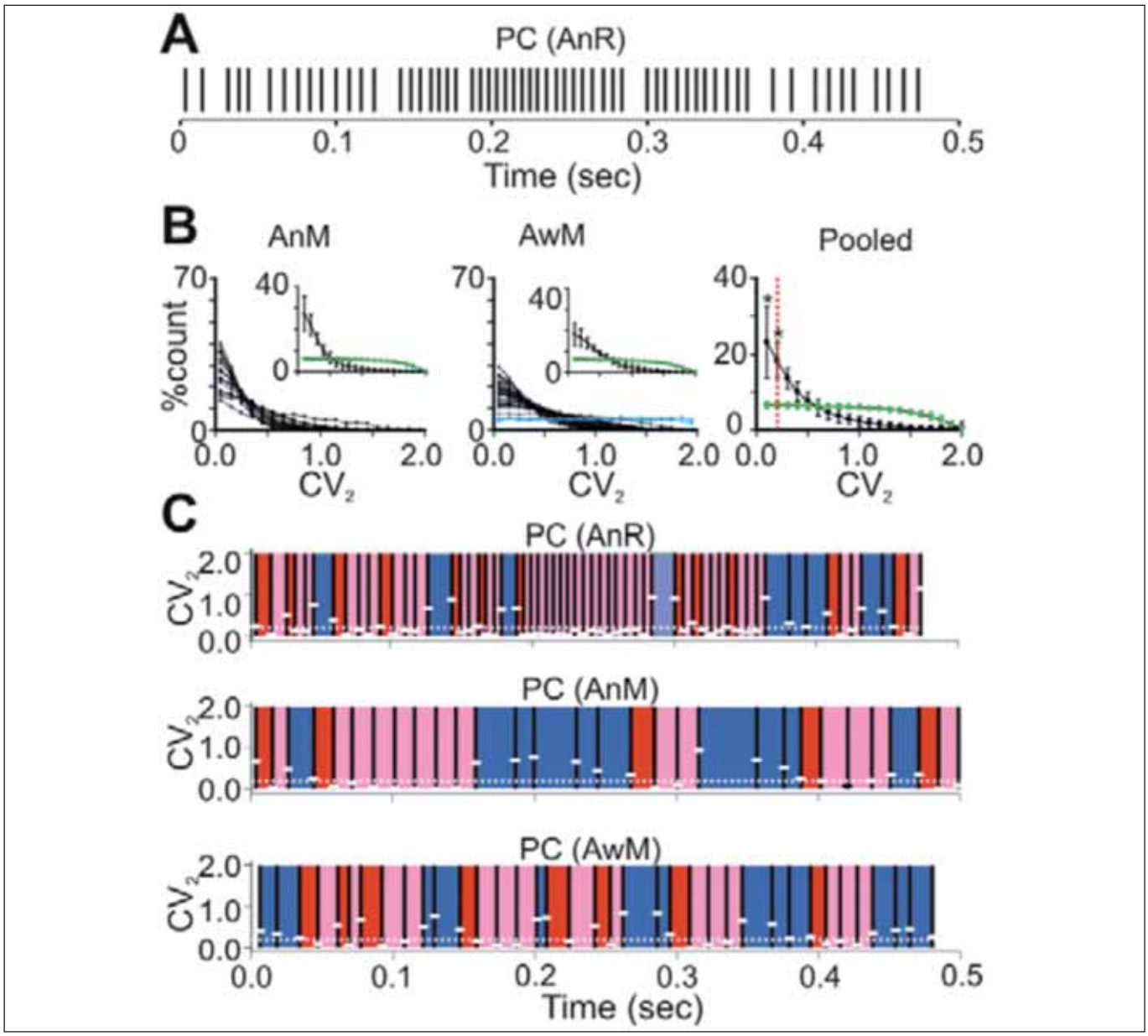

Figure 2: Regular patterns in cerebellar Purkinje cell simple spike trains. (A) Raster plot of PC SS in an anesthetized rat (AnR). (B) CV_2 distributions of SS trains recorded from anesthetized mice (AnM, left), awake mice (AwM, middle, blue: neurons in cerebral motor cortex), and mean of 92 CV_2 distributions (Pooled, right) which were significantly different from those of inhomogeneous Poisson processes with similarly modulated firing rates (red line: CV_2=0.2). Insets and right panel: mean ± s.e.m. (black: PC, green: inhomogeneous Poisson process) (C) Extracting regular spiking patterns by setting CV_2 threshold at 0.2 (white dotted lines). White dashes: CV_2 values calculated from the two surrounding ISIs, red: first ISI of regular patterns, pink: successive ISIs in regular patterns, dark blue: ISIs not belonging to a regular pattern).

We investigated the temporal structure of spontaneous SS trains in data from two sources. Recordings from the cerebellar hemisphere of anaesthetized rats (n = 48) were compared to data from floccular PCs in awake mice (n = 37). Firing rates were similar for both data sets. The *CV* of these spike trains was very high, 3.93 (mean) and 1.39 for rat and mouse PCs respectively,

comparable to previously reported values (Vos et al., 1999; Goossens et al., 2001) and suggesting very irregular firing.

Nevertheless, careful inspection of the spike trains showed clear patterns of regular firing (Fig 2A). We characterized these patterns by computing the CV_2, a measure of short range variability calculated over two consecutive ISIs (Holt et al., 1996). CV_2 showed more regular firing in mice, 0.39 (mean, range 0.25 - 0.73), than in rats, 0.51 (0.13 - 1.07; $p < 0.001$). In both data sets the CV_2 distribution was skewed with a high proportion of low CV_2 values (Fig 2B), suggesting regular spiking patterns. This was very different from CV_2 distributions in other neurons. For example, spontaneous spiking of cortical neurons showed a flat CV_2 distribution (Holt et al., 1996), similar to the realization of a homogeneous Poisson process.

We used a threshold on the measured CV_2 values to isolate the regular spiking patterns. The procedure is illustrated in Fig. 2C: CV_2 was computed for the 2 intervals surrounding each spike. Whenever the CV_2 value was smaller or equal to 0.2 these 2 ISIs were considered part of a regular pattern (pink). If the next ISI also had a CV_2 value lower than 0.2 the following interval belonged to the same pattern, if not the ISI was either single (i.e. not belonging to any pattern, blue) or the start of a new pattern (if the following CV_2 value was lower than 0.2, red). Using this procedure 52% of ISIs in both data sets were classified as belonging to a pattern.

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

In conclusion, we found that the CV_2 can be a measure to isolate regular firing patterns containing 2 to tens of ISIs from spontaneous SS trains in PCs. These patterns contained usually short ISIs, especially if they were long. Support for the functional importance of the regular patterns comes from measuring the effect of tactile stimulation. In the 200 ms following stimulation the proportion of ISIs belonging to regular patterns increased from 54.6% to 78.0%. The expected increase in PC firing frequency following stimulation was expressed as longer regular patterns with faster mean ISIs.

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

Stochastic structure of regular patterns and pauses

Published as Shin et al., *European Journal of Neuroscience* (2007)

To better understand the processes generating regular patterns and pauses we studied their stochastic structure using simple models. Before the detailed description of SS regular patterns and pauses, first stochastic properties of CS firing patterns were studied. We found that (1) there was no serial correlation in CS trains, suggestive of a renewal process, and (2) CS ISIs were a random mixture of two gamma processes, providing an explanatory model using two randomly intermixed oscillatory bands, 0.5 to 1 Hz and 4 to 8 Hz. In the following part, the stochastic structures of SS firing patterns, regular patterns and pauses, were carefully described. First, regular patterns were 20 times more regular than pauses. Although one could simply assume that pauses may be generated by an irregular Poisson process, they were actually generated by rather regular gamma processes. The degrees of regularity quantified by orders of gamma processes revealed that they themselves formed gamma distributions but with different sets of parameters for either regular patterns or pauses. This implies that they were modulated by different processes.

Golgi cell responses to temporal patterns of tactile stimuli

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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.

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. 1). The Golgi cell responded to the shortest stimulus (10 ms) with both early and late ON components (Fig. 1, 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.

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. The ON response profile to a 10Hz frequency stimulus is comparable to the OFF response profile of a 100 ms stimulus duration.

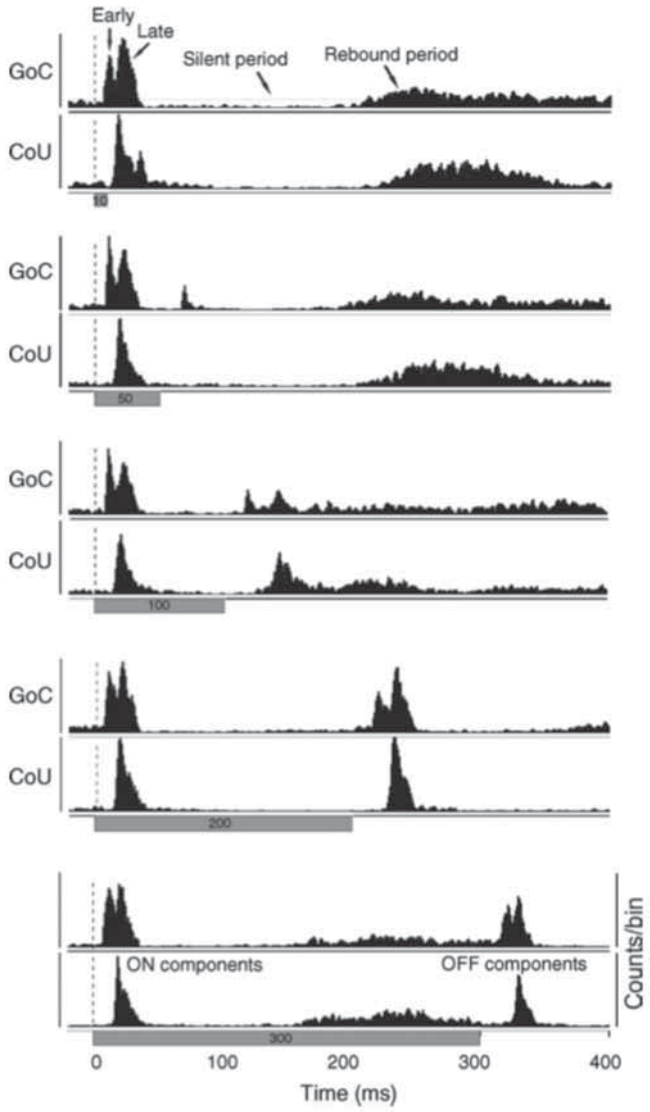

Figure 3: Example of stimulus-evoked responses in a simultaneously recorded cerebellar Golgi cell (GoC) and SI cortical unit (CoU). Stimulus duration indicated by grey bars.

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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Genetic, molecular and cellular mechanisms of cortical development

Background

During the three years 2005-2007, work supported by the FMRE in our laboratory has been focused on: i) The mechanism of action of Reelin and its signaling pathway; (ii) The development and use of in vitro systems to study neuronal migration to the cortex and the formation of the cortical plate; (iii) Role of the seven pass cadherins Celsr3 and Celsr2 in brain development; study of the oncogene p73 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). 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 shown that these antibodies block the reelin signal. We showed that Reelin is cleaved by an enzyme secreted by embryonic neurons and have now evidence that cleavage is required for activity in vivo. This work was published in 2007 (Jossin et al., 2007).

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. His pathway plays a key role in the Reelin-regulated growth of dendrites. This wark was published in 2007 (Jossin and Goffinet, 2007).

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 to screen 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. This work has been published (Zhou et al., 2007).

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

Celsr1-3 are a family of three mammalian gene orthologs of Flamingo (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. Mice with defective Celsr3 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) ((Tissir et al., 2005).

We have made a conditional ("floxed") mutant mouse in which the region deleted in the Celsr3 -/- mice is flanked with loxP sites. Floxed Celsr3 mice are viable and fertile. We are using them in crosses with mice that express the Cre recombinase in forebrain (Foxg1-Cre), in cortical structures (Emx1-Cre), in basal forebrain (Gsh2-Cre and Nkx2.1-Cre) and in dorsal thalamus (Ror1-Cre). This mutant allowed us to perform a genetic dissection of the role of Celsr3 during development of the anterior commissure, of reciprocal thalamocortical fibers, and of subcerebral, particularly the corticospinal tract. The data allow us to propose that Celsr3 is required intrinsically in the neurons of origin of these tracts but also in guidepost cells that guide axons during their progression. Our observations also argue strongly in favor of the "handshake" hypothesis to explain some aspects of thalamocortical wiring. The paper with these important observations has been submitted and is not under review.

We have initiated studies of the mechanism of action of Celsr3, based on a model inspired by the mechanism of PCP in Drosophila. Based on known function of Fzd3 and our in situ study of PCP-like gene expression in mice (Tissir and Goffinet, 2006), we think that Celsr3 may foster interactions between cells that express Fzd3 and Vangl2. We have raised antibodies against Celsr3 and cloned the cDNA coding for Celsr3, Fzd3 and Vangl2, with and without fluorescent tags.

4. Role of oncogene p73 in cortical development

p73 is a gene similar to p53 and p63. It is expressed in two mRNA and protein forms. The full length form is a transcription factor that, like p53, has tumor suppressing activity. The DN form is truncated in its N-terminal moiety, inactive as a transcription factor, but able to dimerize with normal p73, p53 and p63 and behaves as a dominant negative in vitro. DNp73 is highly expressed in Cajal-Retzius neurons, the main producers of Reelin during cortical development, and it has been proposed that it regulates their survival. In order to understand better the function of DNp73 in vivo, we produced mutant mice in which the DNp73 isoform is selectively inactivated, leaving the full length p73 fully active, and in which the Cre and EGFP sequences are knocked in the p73 locus. The production of these animals was difficult to achieve, but they are now available and the mutation is fully validated. Wde have shown that expression of full length p73 is preserved and that both the Cre and EGFP genes are expressed as predicted. This allowed us to show that DNp73 is expressed only in a subset of Cajal-Retzius neurons that originate from the paleoventricle area, whereas the others originate from the cortical hem. We are actively working on the phenotypic characterization of this mutant, and expect to publish a first paper in the autumn 2008.

5. Reviews.

We have participated in reviewing two aspects of brain development, namely the role of Reelin and the issue of thalamocortical wiring (Forster et al., 2006; Price et al., 2006).We have also written a book chapter on cortical development (Tissir, 2007) and a critical review on human specific aspects of brain development (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. Three single-cell studies were started that will provide valuable information for the investigation of the functional interactions between inferior temporal and posterior parietal cortex. A fourth study investigates the interaction between area LIP and the frontal eye fields (FEF), using a paradigm with chronically implanted electrodes in the FEF for microstimulation and simultaneous single-electrode recordings in area LIP during a delayed saccade task.

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 (curvature, aspect ratio, orientation, etc.). Most neurons in the anterior part of LIP (aLIP) were tuned for at least one of the 2D shape dimensions: 101 out of 117 neurons tested (86%) showed significant response differences during the presentation of the 2D shapes. The latency of the neuronal responses was surprisingly short: response onset occurred as early as 30-40 ms after stimulus onset, and differential responses between preferred and nonpreferred shapes emerged 40-50 ms after stimulus onset. To determine to what extent LIP neurons are position invariant for 2D shape we tested 63 LIP neurons tuned for 2D shape with their preferred shape and a nonpreferred shape at 35 positions spaced 2 degrees apart in the contralateral hemifield. Only a limited proportion of the neurons tested (20%) preserved their selectivity at different positions. A large proportion of the neurons (59%) showed responses to the nonpreferred shape at a different position that were equal to or even larger than the response to the preferred shape at the position in the original test, which indicates that the apparent 2D shape selectivity arises from interactions between the position of the stimulus and the shape of the receptive field of the neuron. The remaining neurons (21%) showed complex interactions between shape and position. Hence LIP neurons signal both the 2D shape and the position of stimuli presented during passive fixation. The shape representation in LIP, however, seems to be radically different from the shape representation in the inferior temporal cortex.

A second study investigated the selectivity of posterior parietal neurons for three-dimensional (3D) shapes, in which depth is defined by gradients of binocular disparity. Stimuli and metrics were exactly the same as in our previous studies in the inferior temporal cortex (IT) (Janssen et al., 2000). We recorded 91 single neurons in AIP, an area in the lateral bank of the Intraparietal Sulcus known to be important for grasping, in three passively fixating rhesus monkeys. A large proportion of the neurons (48, 53%) displayed selectivity for the 3D shape of the stimuli that could not be accounted for by the pattern of the monocular responses. The latency of the 3D shape selectivity was 60-70 ms, which is much shorter than that observed in IT (120 ms). Most neurons (36/48, 75%) preserved their selectivity over a number of positions-in-depth, indicating that these neurons are responding to the spatial variation of disparity and not just to differences in the position-in-depth of the stimulus. We determined the sensitivity of posterior parietal neurons by testing 3D shape selective neurons with concave and convex surfaces of varying disparity amplitudes (from 1.3 to 0.03 degrees). The tuning for 3D shape was monotonic in the large majority of the neurons, and

we did not observe a sharp drop in the response between the two smallest amplitudes (-.0.3 and +0.03), as was the case in IT. We also tested 31 AIP neurons with the original 3D shapes and various approximations to these shapes: a wedge stimulus composed of two connected tilted planes, two linear disparity gradients and three different discrete stimuli composed of planar surfaces at different positions in depth. Unlike IT neurons, AIP neurons exhibited considerable selectivity for the discrete approximations, and none of the neurons discriminated between the original and the wedge stimulus. These results indicate that the 3D shape representation in AIP is not only faster and metric, but also coarser compared to IT. Finally, most neurons showed invariance of the 3D shape selectivity for a limited number of positions in the frontoparallel plane and for different sizes (2.5 to 8.5 degrees). Hence the representation of 3D shape in the posterior parietal cortex differs considerably from the representation in the inferior temporal cortex. The data collection for the two previous studies has been completed. Manuscripts have been submitted to the Journal of Neuroscience (the first study) and Science (second study).

In a third study, we prepared two rhesus monkeys for recording and inactivation studies to determine to what extent posterior parietal and inferotemporal neurons interact during the discrimination of 3D shapes. These animals were trained on a 3D-shape discrimination task in which the monkeys have to make an eye movement to the right when the stimulus is a convex surface and to the left when the stimulus is concave. Task difficulty is manipulated by changing the percentage of the dots that give rise to the percept of the curved surface (between 0% and 100% coherence). We determined whether the trial-by-trial variation in the neuronal firing correlated with the monkeys' reported percept of the 3D shape of the stimulus, which can captured by a metric termed the choice probability. Most AIP neurons show positive choice probabilities that are significantly larger than chance. These results indicate that the activity of AIP neurons correlates positively with the reported percept of the animals. We also started simultaneous single-unit and local field potential recordings in AIP and IT during 3D shape discrimination in the same animals. We computed spectral coherence, a measure of synchronization between two continuous signals, and used this statistic to infer functional connectivity between the two regions. The LFP coherence in the gamma-band (i.e. between 60 and 140 Hz) is transiently elevated with respect to the prestimulus period just after stimulus-onset. In contrast, the coherence in the beta-range (i.e. between 15 and 25 Hz) shows a sustained decrease starting ~200ms after stimulus onset. These preliminary findings suggest that neural signals between two highly separated regions can be correlated. In addition, they suggest that the end stages of the dorsal and the ventral visual stream are functionally connected and interact during the discrimination of 3D shape.

Finally, we investigated to what extent the attentional modulation in the activity of LIP neurons originates in the Frontal Eye Fields (FEF). We trained two rhesus monkeys in a delayed saccade task, in which the animals have to make an eye movement to a blue target dot as soon as one out of four simultaneously appearing green dots (the go-cue) changes luminance (either brightening or dimming). We implanted two rhesus monkeys with 40 electrodes in the FEF and prepared the animals for single-cell recordings in area LIP. In at least 30 of the implanted electrodes, microstimulation reliably evoked saccadic eye movements. During the delayed saccade task, either the target or the go-cue could be presented in the receptive field of the LIP neuron, and

microstimulation (currents less than 50% of the threshold for evoking saccades) is applied in 50% of the trials on an FEF electrode in which the response field is either at the same location as the LIP receptive field ('stimulation in' condition) or at a different location ('stimulation out' condition). The artifact that originates from the microstimulation currents is subtracted from the recording signal by means of an artifact zapper (Riverbend Instruments, Birmingham AL). We observed significant decreases in the LIP spike rate in 5 out of 20 neurons (25%) recorded in the 'stimulation in' condition, and in 14 out 31 neurons (45%) recorded in the 'stimulation out' condition, primarily in conditions in which the saccade target appeared in the receptive field of the LIP neuron. The latter result is consistent with the effects of spatial attention. Contrary to previous fMRI findings, we did not find an increase in LIP spike rate during FEF microstimulation when there was no visual stimulus in the receptive field, which could suggest that the fMRI response is based on presynaptic activity. The results of the last two studies are currently being replicated in a second animal.

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Final Report of the Research Group of

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Human Brain Function in Sleep.
Studies in Man by Multimodal Functional Neuroimaging

Our initial objectives were twofold. We wanted to further characterize the neural correlates of human sleep and specify the functional relevance of sleep processes for waking brain function and cognition, at the systems levels, using combined EEG/fMRI acquisitions. These objectives were successfully achieved. Indeed, we were able (i) to examine the neural correlates of sleep using EEG/fMRI; (ii) to characterize experience-dependent changes in brain responses during sleep and assess the influence of post-training sleep and lack of sleep on learning and memory (in a number of different memory systems).

In addition, we were increasingly aware of the functional importance of spontaneous brain activity, not only during sleep, but also during wakefulness. We thus explored if spontaneous waking brain activity modulate the perception of external inputs and the processing of recent memories.

Finally, we realized that the level of arousal could be experimentally manipulated by light, through a novel photoreception system which, in contrast to the classical visual system, is not related to the formation of images of the world. We investigated the non image forming effects of light on human brain function.

1. Characterization of the spontaneous brain activity during normal human sleep

In humans, sleep consists of two drastically different states : non rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. The cellular processes that generate the various NREM sleep oscillations (spindles, K-complexes, delta and slow rhythms) are described in great detail in animals. In humans, positron emission tomography (PET) allows the description of the gross functional neuroanatomy of NREM sleep. However, the poor temporal resolution of this technique does not allow the accurate characterization of the cerebral responses to the transient sleep events described above.

For instance, slow wave activity is related to decrease in cerebral blood flow when measured by PET, whereas we were able to identify increases in activity associated with slow waves during NREM sleep.

> Dang-Vu, T. T., M. Desseilles, et al. (2005). «Cerebral correlates of delta waves during non-REM sleep revisited.» Neuroimage **28**(1): 14-21.
> We aimed at characterizing the neural correlates of delta activity during Non Rapid Eye Movement (NREM) sleep in non-sleep-deprived normal young adults, based on the statistical analysis of a positron emission tomography (PET) sleep data set. One hundred fifteen PET scans were obtained using H(2)(15)O under continuous polygraphic monitoring during stages 2-4 of NREM sleep. Correlations between regional cerebral blood flow (rCBF) and delta power (1.5-4 Hz) spectral density were analyzed using statistical parametric mapping (SPM2). Delta power values obtained at central scalp locations negatively correlated during NREM sleep with rCBF in the ventromedial prefrontal cortex, the basal forebrain, the striatum, the anterior insula, and the precuneus. These regions embrace the set of brain areas in which rCBF

decreases during slow wave sleep (SWS) as compared to Rapid Eye Movement (REM) sleep and wakefulness (Maquet, P., Degueldre, C., Delfiore, G., Aerts, J., Peters, J.M., Luxen, A., Franck, G., 1997. Functional neuroanatomy of human slow wave sleep. J. Neurosci. 17, 2807-S2812), supporting the notion that delta activity is a valuable prominent feature of NREM sleep. A strong association was observed between rCBF in the ventromedial prefrontal regions and delta power, in agreement with electrophysiological studies. In contrast to the results of a previous PET study investigating the brain correlates of delta activity (Hofle, N., Paus, T., Reutens, D., Fiset, P., Gotman, J., Evans, A.C., Jones, B.E., 1997. Regional cerebral blood flow changes as a function of delta and spindle activity during slow wave sleep in humans. J. Neurosci. 17, 4800-4808), in which waking scans were mixed with NREM sleep scans, no correlation was found with thalamus activity. This latter result stresses the importance of an extra-thalamic delta rhythm among the synchronous NREM sleep oscillations. Consequently, this rCBF distribution might preferentially reflect a particular modulation of the cellular processes involved in the generation of cortical delta waves during NREM sleep.

Dang Vu, T. T. et al. « Spontaneous Neuronal Activity during Human Slow Wave Sleep : An EEG/fMRI Study." (submitted)

In animals, intracellular recordings show that deep non rapid eye movement (NREM) sleep is associated with substantial neuronal activity. A slow oscillation takes place synchronously in large populations of cortical neurons and alternates prolonged depolarization phases, associated with intense firing, and periods of hyperpolarization during which neurons are silent(Steriade et al., 1993b; Steriade et al., 1993a; Steriade et al., 2001). In contrast, human deep NREM has been systematically associated with global and regional decreases in brain metabolism and blood flow, relative to wakefulness or REM sleep(Maquet, 2000). Nevertheless, the cerebral correlates of slow oscillation during NREM sleep have never been characterized in humans. Here, we assessed the changes in regional brain activity consistently associated with slow oscillation, detected as high amplitude slow waves(Hobson et al., 2000; Molle et al., 2002; Massimini et al., 2004), using simultaneous electroencephalography (EEG) and event-related functional magnetic resonance imaging (fMRI) during deep NREM sleep, in 14 non-sleep deprived normal human volunteers. Significant increases in activity were identified not only in the cortex and thalamus but also in the ponto-mesencephalic tegmentum, posterior hypothalamus, cerebellum and basal ganglia. Within the cortex, slow oscillation was consistently associated with increased activity in distributed although discrete areas. These regions not only involved prefrontal association cortex but also primary cortices, as well as limbic (hippocampus and parahippocampal gyrus) and paralimbic regions. No significant activity decrease was observed. Human deep NREM sleep is not, as usually thought, a state of brain quiescence, but is an active state during which brain activity is synchronized to the slow oscillation. Slow oscillation appears as a global brain phenomenon which shapes regional brain activity and is in position to profoundly influence cerebral processing of incoming information and past experience.

Fifty percents of sleep time is spent in stage 2 sleep, mainly characterized by spindles and K complexes. Two types of spindles are observed in humans but their neural correlates are unknown. Using EEG/fMRI, we were able to demonstrate the existence of these 2 spindle types and the associated cerebral responses.

Schabus, M., T. T. Dang-Vu, et al. (2007). «Hemodynamic cerebral correlates of sleep spindles during human non-rapid eye movement sleep.» Proc Natl Acad Sci U S A 104(32): 13164-9.
In humans, some evidence suggests that there are two different types of spindles during sleep, which differ by their scalp topography and possibly some aspects of their regulation. To test for the existence of two different spindle types, we characterized the activity associated with slow (11-13 Hz) and fast (13-15 Hz) spindles, identified as discrete events during non-rapid eye movement sleep, in non-sleep-deprived human volunteers, using simultaneous electroencephalography and functional MRI. An activation pattern common to both spindle types involved the thalami, paralimbic areas (anterior cingulate and insular cortices), and superior temporal gyri. No thalamic difference was detected in the direct comparison between slow and fast spindles although some thalamic areas were preferentially activated in relation to either spindle type. Beyond the common activation pattern, the increases in cortical activity differed significantly between the two spindle types. Slow spindles were associated with increased activity in the superior frontal gyrus. In contrast, fast spindles recruited a set of cortical regions involved in sensorimotor processing, as well as the mesial frontal cortex and hippocampus. The recruitment of partially segregated cortical networks for slow and fast spindles further supports the existence of two spindle types during human non-rapid eye movement sleep, with potentially different functional significance.

2. Offline memory processing during sleep – Hippocampal-dependent memories

A number of experiments in rodents suggest that during sleep, neural activity patterns related to the experiences of the previous awake period are replayed during sleep. We were the first to demonstrate that the distribution of regional brain activity is likewise modified by previous experience in humans:

Peigneux, P., S. Laureys, et al. (2004). «Are spatial memories strengthened in the human hippocampus during slow wave sleep?» Neuron **44**(3): 535-45.
In rats, the firing sequences observed in hippocampal ensembles during spatial learning are replayed during subsequent sleep, suggesting a role for posttraining sleep periods in the offline processing of spatial memories. Here, using regional cerebral blood flow measurements, we show that, in humans, hippocampal areas that are activated during route learning in a virtual town are likewise activated during subsequent slow wave sleep. Most importantly, we found that the amount of hippocampal activity expressed during slow wave sleep positively correlates with the improvement of performance in route retrieval on the next day. These findings suggest that learning-dependent modulation in hippocampal activity during human sleep reflects the offline processing of recent episodic and spatial memory traces, which eventually leads to the plastic changes underlying the subsequent improvement in performance.

In addition, we showed in two different tasks that sleep deprivation during the first post-encoding night hinders the plastic processes which usually underpin memory consolidation during sleep. With the second task, we further showed that sleep deprivation may have remote effects, up to 6 month after encoding.

Orban, P., G. Rauchs, et al. (2006). «Sleep after spatial learning promotes covert reorganization of brain activity.» Proc Natl Acad Sci U S A **103**(18): 7124-9.
Sleep promotes the integration of recently acquired spatial memories into cerebral networks for the long term. In this study, we examined how sleep deprivation hinders this consolidation process. Using functional MRI, we mapped regional cerebral activity during place-finding navigation in a virtual town, immediately after learning and 3 days later, in subjects either allowed regular sleep (RS) or totally sleep-deprived (TSD) on the first posttraining night. At immediate and delayed retrieval, place-finding navigation elicited increased brain activity in an extended hippocampo-neocortical network in both RS and TSD subjects. Behavioral performance was equivalent between groups. However, striatal navigation-related activity increased more at delayed retrieval in RS than in TSD subjects. Furthermore, correlations between striatal response and behavioral performance, as well as functional connectivity between the striatum and the hippocampus, were modulated by posttraining sleep. These data suggest that brain activity is restructured during sleep in such a way that navigation in the virtual environment, initially related to a hippocampus-dependent spatial strategy, becomes progressively contingent in part on a response-based strategy mediated by the striatum. Both neural strategies eventually relate to equivalent performance levels, indicating that covert reorganization of brain patterns underlying navigation after sleep is not necessarily accompanied by overt changes in behavior.

Gais, S., G. Albouy, et al. (2007). «Sleep transforms the cerebral trace of declarative memories.» Proc Natl Acad Sci U S A **104**(47): 18778-83.
After encoding, memory traces are initially fragile and have to be reinforced to become permanent. The initial steps of this process occur at a cellular level within minutes or hours. Besides this rapid synaptic consolidation, systems consolidation occurs within a time frame of days to years. For declarative memory, the latter is presumed to rely on an interaction between different brain regions, in particular the hippocampus and the medial prefrontal cortex (mPFC). Specifically, sleep has been proposed to provide a setting that supports such systems consolidation processes, leading to a transfer and perhaps transformation of memories. Using functional MRI, we show that postlearning sleep enhances hippocampal responses during recall of word pairs 48 h after learning, indicating intrahippocampal memory processing during sleep. At the same time, sleep induces a memory-related functional connectivity between the hippocampus and the mPFC. Six months after learning, memories activated the mPFC more strongly when they were encoded before sleep, showing that sleep leads to long-lasting changes in the representation of memories on a systems level.

3. Offline memory processing during sleep – Emotional memories

The consolidation of emotional declarative memories probably requires a functional interplay between the amygdala, the hippocampus and neocortical stores. Sleep is believed to take part in the consolidation of emotional memories, although the neural correlates of sleep-dependent emotional memory consolidation are still unknown.

In a first study, we studies the effect of an immediate recall. We were able to show that the correct recollection of emotional memories involves the locus coeruleus, the main noradrenergic nucleus of the brain. However, the recruitment of LC was conditional upon the level of arousal achieved during encoding. This finding suggests that recollection of emotional memories relies in part on the restoration of the arousal produced at encoding.

> Sterpenich, V., A. D'Argembeau, et al. (2006). «The locus ceruleus is involved in the successful retrieval of emotional memories in humans.» J Neurosci **26**(28): 7416-23.
> Emotional memories are better remembered than neutral ones. The amygdala is involved in this enhancement not only by modulating the hippocampal activity, but possibly also by modulating central arousal. Using functional magnetic resonance imaging, we analyzed the retrieval of neutral faces encoded in emotional or neutral contexts. The pupillary size measured during encoding was used as a modulator of brain responses during retrieval. The interaction between emotion and memory showed significant responses in a set of areas, including the amygdala and parahippocampal gyrus. These areas responded significantly more for correctly remembered faces encoded in an emotional, compared with neutral, context. The same interaction conducted on responses modulated by the pupillary size revealed an area of the dorsal tegmentum of the ponto-mesencephalic region, consistent with the locus ceruleus. Moreover, a psychophysiological interaction showed that amygdalar responses were more tightly related to those of the locus ceruleus when remembering faces that had been encoded in an emotional, rather than neutral, context. These findings suggest that the restoration of a central arousal similar to encoding takes part in the successful retrieval of neutral events learned in an emotional context.

We then probed the effects of sleep and lack of sleep on the systems consolidation of emotional episodic memories. We were able to show that depending on whether or not sleep is allowed on the first post-encoding night, different response patterns were observed during recall 72 hours later, suggesting a significant effect of sleep on emotional memory consolidation.

> Sterpenich, V., G. Albouy, et al. (2007). "Sleep-Related Hippocampo-Cortical Interplay during Emotional Memory Recollection." PLoS Biol **5**(11): e282.
> Emotional events are usually better remembered than neutral ones. This effect is mediated in part by a modulation of the hippocampus by the amygdala. Sleep plays a role in the consolidation of declarative memory. We examined the impact of sleep and lack of sleep on the consolidation of emotional (negative and positive) memories at the macroscopic systems level. Using functional MRI (fMRI), we compared the neural correlates of successful recollection by humans of emotional and neutral stimuli, 72 h after encoding,

with or without total sleep deprivation during the first post-encoding night. In contrast to recollection of neutral and positive stimuli, which was deteriorated by sleep deprivation, similar recollection levels were achieved for negative stimuli in both groups. Successful recollection of emotional stimuli elicited larger responses in the hippocampus and various cortical areas, including the medial prefrontal cortex, in the sleep group than in the sleep deprived group. This effect was consistent across subjects for negative items but depended linearly on individual memory performance for positive items. In addition, the hippocampus and medial prefrontal cortex were functionally more connected during recollection of either negative or positive than neutral items, and more so in sleeping than in sleep-deprived subjects. In the sleep-deprived group, recollection of negative items elicited larger responses in the amygdala and an occipital area than in the sleep group. In contrast, no such difference in brain responses between groups was associated with recollection of positive stimuli. The results suggest that the emotional significance of memories influences their sleep-dependent systems-level consolidation. The recruitment of hippocampo-neocortical networks during recollection is enhanced after sleep and is hindered by sleep deprivation. After sleep deprivation, recollection of negative, potentially dangerous, memories recruits an alternate amygdalo-cortical network, which would keep track of emotional information despite sleep deprivation.

4. Offline memory processing of motor sequence memories during post-training wakefulness and sleep

Consolidation of motor memories is believed to unfold in two separate steps : a stabilization phase during which motor skill learning becomes resistant to interference and a sleep-dependent proper consolidation phase characterized by a significant gain in performance. We demonstrated that within the hour after training, performance was increased as much as after 24 hours. We believe that this novel early step, which we called 'early boost', represent an early structuring of the motor memory.

Hotermans, C., P. Peigneux, et al. (2006). «Early boost and slow consolidation in motor skill learning.» Learn Mem **13**(5): 580-3.

Motorskill learning is a dynamic process that continues covertly after training has ended and eventually leads to delayed increments in performance. Current theories suggest that this off-line improvement takes time and appears only after several hours. Here we show an early transient and short-lived boost in performance, emerging as early as 5-30 min after training but no longer observed 4 h later. This early boost is predictive of the performance achieved 48 h later, suggesting its functional relevance for memory processes.

The various steps of on motor skill consolidation have been exclusively shown using manual tasks. We generalized these findings in showing that learning a oculomotor serial reaction time task follows the same time course, characterized by an early boost, a stabilization phase and a consolidation phase. Intriguingly, only the latter was associated with a gain in performance specific to the learned sequence.

Albouy, G., P. Ruby, et al. (2006). «Implicit oculomotor sequence learning in humans: Time course of offline processing.» Brain Res **1090**(1): 163-171.
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 min, 5 h or 24 h. 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 min after training, a significant gain in oculomotor performance was observed irrespective of the sequence learning. This gain was no longer present after 5 h. Remarkably, a gain in performance specific to the learned sequence emerged only 24 h 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.

We then embarked on a large-scale fMRI study conducted on about a hundred subjects, during which we described the neural correlates of the 3 phases of motor skill offline processing. Importantly, we found that in addition to the striatum, the hippocampus is also involved in motor sequence memory consolidation.

Albouy, G., E. Balteau et al. (Accepted for publication in Neuron) Both the Hippocampus and Striatum Are Involved in Oculomotor Sequence Memory Consolidation.
Functional magnetic resonance imaging (fMRI) was used to explore the cerebral correlates of implicit oculomotor sequence learning, during training and a single test session taking place 30 minutes, 5 or 24 hours later. During training, learning-related responses were observed in hippocampus and caudate nucleus. Both responses were linearly related to the gain in performance observed overnight, but not over the day. A gain in performance specific to the learned sequence tended to emerge only after 24 hours. At that time, learning-related responses in hippocampus and striatum were significantly larger than at 30 minutes and 5 hours respectively. Additionally, the competitive interaction observed between hippocampus and striatum during training became cooperative overnight. These results suggest that responses in hippocampus and striatum during initial training condition the overnight memory processing. The latter is eventually associated with a change in the functional interaction of cerebral responses underpinning motor sequence learning.

5. Spontaneous waking brain activity modulates perception and offline memory processing
Classically, the brain's operations are considered as essentially reflexive and mainly driven by external stimuli. In this perspective, brain function is predominantly geared to interpreting incoming stimuli and programming motor output. Another view posits that the bulk of brain's activity is

intrinsic, spontaneous (i.e., it emerges in the absence of any identified external stimulus), and essentially aims at maintaining and processing information. The intrinsic brain activity profoundly is known to modulates neural responses to external stimuli. In a first experiment, we tested the hypothesis that, at the macroscopic systems level, spontaneous human brain activity modulates the conscious perception of noxious stimuli.

> Boly, M., E. Balteau, et al. (2007). «Baseline brain activity fluctuations predict somatosensory perception in humans.» Proc Natl Acad Sci U S A **104**(29): 12187-92.
> In perceptual experiments, within-individual fluctuations in perception are observed across multiple presentations of the same stimuli, a phenomenon that remains only partially understood. Here, by means of thulium-yttrium/aluminum-garnet laser and event-related functional MRI, we tested whether variability in perception of identical stimuli relates to differences in prestimulus, baseline brain activity. Results indicate a positive relationship between conscious perception of low-intensity somatosensory stimuli and immediately preceding levels of baseline activity in medial thalamus and the lateral frontoparietal network, respectively, which are thought to relate to vigilance and "external monitoring." Conversely, there was a negative correlation between subsequent reporting of conscious perception and baseline activity in a set of regions encompassing posterior cingulate/precuneus and temporoparietal cortices, possibly relating to introspection and self-oriented processes. At nociceptive levels of stimulation, pain-intensity ratings positively correlated with baseline fluctuations in anterior cingulate cortex in an area known to be involved in the affective dimension of pain. These results suggest that baseline brain-activity fluctuations may profoundly modify our conscious perception of the external world.

In a second experiment, we assessed whether spontaneous brain activity induced by recent experiences modulates responses to an unrelated cognitive challenge. This peculiar experimental design allowed us to characterized the memory trace in the waking brain, without the confounds of simultaneous recall processes. Intriguingly, we found that the learning-dependent activity is not only maintained during subsequent waking period but that it is changing over time, suggesting an early off line processing of memories during immediate post-training wakefulness.

> Peigneux, P., P. Orban, et al. (2006). «Offline persistence of memory-related cerebral activity during active wakefulness.» PLoS Biol **4**(4): e100.
> 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 post-training 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 post-training wakefulness before sleep, while the brain is actively engaged in other cognitive activities. We show, using functional magnetic resonance imaging, that brain activity elicited during a new learning episode modulates brain responses to an unrelated cognitive task, during the waking period following the end of training. This post-training activity evolves in learning-related cerebral structures, in which functional connections with other brain regions are gradually established or reinforced. It also correlates with

behavioral performance. These processes follow a different time course for hippocampus-dependent and hippocampus-independent memories. Our experimental approach allowed the characterization of 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 has already extensively processed recent memories during the first hours of post-training wakefulness, even when simultaneously coping with unrelated cognitive demands.

6. Modulating arousal : characterization of the functional role of non classical photoreception in humans

Light profoundly affects human physiology, in addition to vision. Exposure to light is the primary synchronizer of circadian rhythms, but light also induces acute physiological responses. These responses involve functions not directly related to vision and include the modulation of the sleep/wake cycle, thermoregulation, endocrine functions, alertness, and performances. Animal and human studies demonstrated that a *nonvisual* photoreception system most sensitive to shorter wavelength light (~470nm; blue light) mediates these effects. This system recruits the classical retinal photoreceptors (rods and cones) and intrinsically photosensitive retinal ganglion cells (RGC) expressing melanopsin. These RGC project to numerous nuclei of the brainstem, hypothalamus, thalamus, and to cortical structures, an anatomical connectivity which suggests that the nonvisual system can influence many brain functions. However, beyond the direct retinal projections, little is known on the other brain structures involved. In a first positron emission tomography (PET) study we showed that night time bright white light exposure (>8000lux) modulates the brain activity related to an attentional task.

Perrin, F., P. Peigneux, et al. (2004). «Nonvisual responses to light exposure in the human brain during the circadian night.» Curr Biol **14**(20): 1842-6.

The brain processes light information to visually represent the environment but also to detect changes in ambient light level. The latter information induces non-image-forming responses and exerts powerful effects on physiology such as synchronization of the circadian clock and suppression of melatonin. In rodents, irradiance information is transduced from a discrete subset of photosensitive retinal ganglion cells via the retinohypothalamic tract to various hypothalamic and brainstem regulatory structures including the hypothalamic suprachiasmatic nuclei, the master circadian pacemaker. In humans, light also acutely modulates alertness, but the cerebral correlates of this effect are unknown. We assessed regional cerebral blood flow in 13 subjects attending to auditory and visual stimuli in near darkness following light exposures (>8000 lux) of different durations (0.5, 17, 16.5, and 0 min) during the biological night. The bright broadband polychromatic light suppressed melatonin and enhanced alertness. Functional imaging revealed that a large-scale occipito-parietal attention network, including the right intraparietal sulcus, was more active in proportion to the duration of light exposures preceding the scans. Activity in the hypothalamus decreased in proportion to previous illumination. These findings have important implications for understanding the effects of light on human behavior.

We then turn to fMRI and daytime experiments. The first fMRI experiment assessed the brain responses to an auditory attentional task before and after exposure to a 21min bright white light (>7000lux). Light-induced improvement in subjective alertness was linearly related to an increased responsiveness in the thalamus. In addition, light enhanced responses in a set of cortical areas involved in the task, 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 first findings suggest that light can modulate the activity of subcortical structures involved in alertness, thereby dynamically promoting cortical activity in networks involved in ongoing nonvisual cognitive processes.

> Vandewalle, G., E. Balteau, et al. (2006). «Daytime light exposure dynamically enhances brain responses.» Curr Biol **16**(16): 1616-21.
> In humans, light enhances both alertness and performance during nighttime and daytime and influences regional brain function . These effects do not correspond to classical visual responses but involve a non-image forming (NIF) system, which elicits greater endocrine, physiological, neurophysiological, and behavioral responses to shorter light wavelengths than to wavelengths geared toward the visual system . During daytime, the neural changes induced by light exposure, and their time courses, are largely unknown. With functional magnetic resonance imaging (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 a short exposure to a bright white light. Light-induced improvement in subjective alertness was linearly related to responses in the posterior thalamus. In addition, light enhanced responses in a set of cortical areas supporting attentional oddball effects, and it prevented decreases of activity otherwise observed during continuous darkness. Responses to light were remarkably dynamic. They declined within minutes after the end of the light stimulus, following various region-specific time courses. These findings suggest that light can modulate activity of subcortical structures involved in alertness, thereby dynamically promoting cortical activity in networks involved in ongoing nonvisual cognitive processes.

In the second fMRI study, we used short (18min) exposure to blue (470nm) or green (550nm) monochromatic light, based on the fact that melanopsin expressing photosensitive retinal ganglion cells have a peak sensitivity around 470 nm. Blue light typically enhanced brain responses or, at least, prevented the decline otherwise observed during green light exposure in frontal and parietal cortices implicated in working memory, and in the thalamus. These results imply that monochromatic light can affect cognitive functions almost instantaneously and suggest that these effects are mediated by a melanopsin-based photoreceptor system.

> Vandewalle, G., S. Gais, et al. (2007). «Wavelength-Dependent Modulation of Brain Responses to a Working Memory Task by Daytime Light Exposure.» Cereb Cortex **17**(12): 2788-95.
> In addition to classical visual effects, light elicits nonvisual brain responses, which profoundly influence physiology and behavior. These effects are mediated in part by melanopsin-expressing light-sensitive ganglion cells that, in contrast to the classical photopic system that is maximally sensitive to green light (550 nm), is very sensitive to blue light (470-480 nm). At

present, there is no evidence that blue light exposure is effective in modulating nonvisual brain activity related to complex cognitive tasks. Using functional magnetic resonance imaging, we show that, while participants perform an auditory working memory task, a short (18 min) daytime exposure to blue (470 nm) or green (550 nm) monochromatic light (3 x 10(13) photons/cm(2)/s) differentially modulates regional brain responses. Blue light typically enhanced brain responses or at least prevented the decline otherwise observed following green light exposure in frontal and parietal cortices implicated in working memory, and in the thalamus involved in the modulation of cognition by arousal. Our results imply that monochromatic light can affect cognitive functions almost instantaneously and suggest that these effects are mediated by a melanopsin-based photoreceptor system.

In the last experiment, we used repeated very short (50s) monochromatic violet (430nm), blue (473nm), and green (527nm) lights of equal photon flux. Wavelength-sensitive brain responses were still detectable at light onsets and during the task. Results support a prominent contribution of melanopsin RGC to nonvisual brain responses within the very first seconds of a light exposure in brain areas involved in arousal regulation and in the task. Results suggest the implication of the brainstem and of the thalamus in establishing nonvisual responses to light.

Vandewalle, G., C. Schmidt, et al. (2007). «Brain responses to violet, blue, and green monochromatic light exposures in humans: prominent role of blue light and the brainstem.» PLoS ONE **2**(11): e1247.

BACKGROUND: Relatively long duration retinal light exposure elicits nonvisual responses in humans, including modulation of alertness and cognition. These responses are thought to be mediated in part by melanopsin-expressing retinal ganglion cells which are more sensitive to blue light than violet or green light. The contribution of the melanopsin system and the brain mechanisms involved in the establishment of such responses to light remain to be established. METHODOLOGY/PRINCIPAL FINDINGS: We exposed 15 participants to short duration (50 s) monochromatic violet (430 nm), blue (473 nm), and green (527 nm) light exposures of equal photon flux (10(13)ph/cm(2)/s) while they were performing a working memory task in fMRI. At light onset, blue light, as compared to green light, increased activity in the left hippocampus, left thalamus, and right amygdala. During the task, blue light, as compared to violet light, increased activity in the left middle frontal gyrus, left thalamus and a bilateral area of the brainstem consistent with activation of the locus coeruleus. CONCLUSION/SIGNIFICANCE: These results support a prominent contribution of melanopsin-expressing retinal ganglion cells to brain responses to light within the very first seconds of an exposure. The results also demonstrate the implication of the brainstem in mediating these responses in humans and speak for a broad involvement of light in the regulation of brain function.

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Hereafter, we provide a comprehensive list of the research work which has benefited from the support of the Queen Elisabeth Medical Foundation between 2004 and 2007.

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Final 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

I.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) and most of its effects are triggered by AT_1 receptor activation. However, shorter fragments such as Ang-(1-7), Ang III and Ang IV have been shown to be bioactive as well. In this respect, the hexapeptide Ang IV sparked great interest because of its facilitatory role in memory acquisition and retrieval.

Ang IV binds also to the AT_1 receptors but only with low affinity. Yet, as most of its physiological effects are already observed at nanomolar concentrations, it is generally accepted that they are mediated via a novel angiotensin receptor subtype: the "AT_4 receptor" (de Gasparo et al., 1995, 2000). 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) and Norleucinal (Chai et al., 2000, Albiston et al., 2001). 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 (Møeller et al., 1997). Among them LVV-H7 displays the highest affinity for the AT_4 receptors.

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) (Albiston et al., 2001). IRAP is a type II integral membrane protein homologous to aminopeptidase N (AP-N), and other Zn^{2+}-dependent enzymes of the gluzincin aminopeptidase family (Rogi et al., 1996, for review see Vauquelin et al., 2002).

I.2. Role of Ang IV in memory and learning

Initial interest in Ang IV originated from its ability to increase memory acquisition and recall in passive and conditioned avoidance response studies (Braszko et al., 1998, Wright et al., 1993, 1996, Tchekalarova et al., 2001). Intracerebroventricular (i.c.v.) administration of the AT_4 agonist Nle^{l}-Ang IV facilitated the ability 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 (Wright et al., 1999). Divalinal-Ang IV also counteracted scopolamine-induced disruption of spatial learning (Pedersen et al., 1998). Moreover, Ang IV and LVV-H7 facilitated potassium-evoked acetylcholine release from rat hippocampal slices (Lee et al., 2001), 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* (Kramar et al., 2001) and *in vivo* (Wayner et al., 2001). Moreover, autoradiographic studies revealed that AT_4 binding

sites are prominent in brain structures important to cognitive processing, including hippocampus (Miller-Wing et al., 1993).

I.3. Working hypotheses and specific aims of the project

The above 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 (Albiston et al., 2004). We are performing a critical evaluation of the working hypotheses that IRAP is indeed the AT_4 receptor, and that the IRAP enzyme/AT_4 receptor system represents a 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 (Shipp and Look, 1993) 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 (Gaetaniello et al., 1998, Navarette Santos et al., 2000). Therefore we investigated the capability of the IRAP enzyme/AT_4 receptor system, after binding with Ang IV, to trigger intracellular signalling pathways in neuronal cells.

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 was 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 were performed in rats, wild-type mice and IRAP knock-out mice to give a 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 (Kovacs et al., 1994, Matsuoka et al., 1995, Alescio-Lautier et al., 2000), 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 (Albiston et al., 2001). In this project, we investigated if inhibition of IRAP by Ang IV indeed resulted *in vivo* in an increased extracellular concentration of these neuropeptides of interest. Moreover, selective oxytocin, vasopressin and somatostatin receptor antagonists were 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" system as well as the mechanisms of action of Ang IV within learning and memory processes.

In the past 4 years, we acquired a better understanding of, first, the presence and properties of the IRAP system in cell lines and, second, of the in vivo effects and mechanisms of action

of Ang IV in the central nervous system. A combination of in vitro and in vivo approaches was essential to this end and proved to be successful. Part of the work was dedicated to the study of the involvement of the IRAP system in pathophysiological conditions, such as epileptic seizures and cognitive decline, to evaluate the possible involvement of AT_1 receptors in the observed physiological effects. In the next sections we provide an overview of our findings.

II. Characterisation and function of the IRAP enzyme/AT_4 receptor system *in vitro*

II.1. Endogeneous expression of IRAP in Chinese hamster ovary (CHO-K1) cells and its modulation by metal ion chelators.

IRAP or cystinyl aminopeptidase (EC 3.4.11.3), a membrane-associated zinc-dependent metallopeptidase of the M1 family, displays high affinity for Ang IV and it was proposed to represent the AT_4 receptor. In our first experimental paper related to the IRAP enzyme/AT_4 receptor system (Demaegdt et al., 2004a), **we identified and characterised the presence of endogenous IRAP in membranes from CHO-K1** cells by binding studies with [^{125}I]Ang IV and by measuring the cleavage of the substrate L-leucine-p-nitroanilide. Binding was displaced with high potency by the known " AT_4 receptor" ligands (Ang IV > Divalinal1-Ang IV = LVV-hemorphin-7 (LVV-H7) = LVV-hemorphin-6 > Ang (3-7) > Ang III > Ang (4-8)) but not by AT_1/AT_2 receptor antagonists. High affinity binding of [^{125}I]Ang IV required the presence of metal chelators and as a consequence, ligands such as Ang IV and LVV-hemorphin-7 displayed higher potency in binding studies compared to enzyme assays. These pharmacological properties match those previously reported for the recombinantly-expressed human IRAP in embryonic kidney cells (Albiston et al, 2001).

Membranes of CHO-K1 cells were subsequently used to study the opposite modulation of enzyme activity and [^{125}I]Ang IV binding to IRAP by divalent cation chelators (Demaegdt et al., 2004b). Whereas ethylene diamine tetraacetic acid (EDTA) or ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) alone only slightly affected the enzyme activity, 1,10-phenanthroline produced a complete and concentration-dependent inhibition. Interestingly EDTA or EGTA enhanced the inhibitory effect of 1,10-phenanthroline. Two-site analysis of the corresponding inhibition curves revealed that EDTA and EGTA converted enzymes with low sensitivity towards 1,10-phenanthroline into enzymes with high sensitivity. The combined inhibition by EDTA and 1,10-phenanthroline could be prevented and reversed by addition of Zn^{2+}. In contrast, specific binding of [^{125}I]Ang IV was enhanced in the presence of 1,10-phenanthroline. Binding was only slightly affected by EDTA or EGTA alone. Furthermore, the stimulatory effect of 1,10-phenanthroline was potentiated by EDTA as well as EGTA. In the presence of EDTA and 1,10-phenanthroline, specific [^{125}I]Ang IV binding was completely inhibited by Zn^{2+}. These data confirmed that divalent cations such as Zn^{2+} are essential for the enzyme activity of IRAP and inhibitory for [^{125}I]Ang IV binding. Moreover, these data demonstrated that, **in addition to the binding site for Zn^{2+} in the catalytic site, IRAP also bears a novel regulatory divalent cation binding site**.

II.2. Pharmacological distinction between IRAP and AP-N in different cell lines and high affinity binding of Ang IV to only the IRAP apoenzyme

Due to its high affinity for [^{125}I]Ang IV, IRAP has been assigned as the AT_4 receptor. Since the aminopeptidase N (AP-N) activity is also susceptible to inhibition by Ang IV, it might represent an additional target for this peptide. The possibility 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 both more potent IRAP-inhibitors. 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. By comparing the enzyme activity of IRAP and AP-N it appeared that all tested AT_4 ligands selectively inhibit IRAP. (Demaegdt et al., 2006). With this respect **Ang IV has a 20 fold selectivity for recombinant human IRAP as compared with human and porcine AP-N**. These differences were used to explore the presence of IRAP and/or AP-N in different cell lines, e.g. CHO-K1, COS-7, HEK293, SK-N-MC and MDBK. We thus provided evidence that **mainly IRAP is present** in the above mentioned cell lines and that CHO-K1 cells display the highest level of this enzyme (Demaegdt et al., 2006).

IRAP and AP-N have one Zn^{2+} binding motif and are members 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 (see part II.1.), 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. Similar results for AP-N as for IRAP in II.1. were found. Moreover, for both enzymes, competition between 1,10-phenanthroline and the substrate only took place in the presence of EDTA. And, the reversion by Zn^{2+} of the inhibitory effect of EDTA plus 1,10-phenanthroline was potentiated when Ca^{2+} was added to the incubation medium.

These findings are novel 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 apoenzymes (i.e. enzymes lacking the catalytic Zn^{2+}) is that **high affinity [^{125}I]-Ang IV binding exclusively takes place to the apoenzyme form of IRAP**. The selectivity of this process (i.e. no binding to AP-N) **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 (Laeremans et al., 2005; Vanderheyden et al., 2006).

In collaboration with Dr. S. Chakravarthy (Dept of Molecular Visual Plasticity, Netherlands Ophthalmic Research Institute, Amsterdam) we obtained P40H1 which is **a hippocampal cell neuronal line**, established from a postnatal day 40 adult H-2Kb-tsA58 transgenic mouse hippocampus. P40H1 cells harbor the temperature-sensitive SV40 T-antigen, which makes it immortal at the permissive temperature of 34 °C. Preliminar [125I]-Ang IV binding data revealed that this cell line display **a**

high level of endogenous IRAP/AT4 receptors. Currently we are investigating whether Ang IV (as was already shown for CHOK1 cells) is capable of increasing [3H]-thymidine incorporation (to measure cell division) and/or triggering intracellular signalling pathways in these cells.

II.3. IRAP/AT_4 mediated intracellular signalling

We have investigated whether IRAP/AT_4 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. While, until now Ang IV was not found to cause an effect on intracellular Ca^{2+} concentration, it enhanced **[^{3}H]-thymidine incorporation in CHO-K1 cells**, an effect that was not blocked by $AT_{1/2}$ receptor antagonists. 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 (Esteban et al., 2005). It is not yet clear whether Ang IV also regulates these genes in neuronal cells

II.4. Subcellular localisation and translocation of IRAP

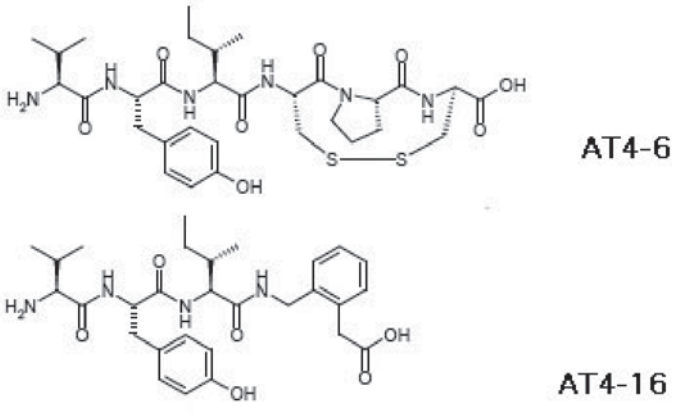
In adipocytes and muscle cells, it is well known that IRAP co-localises with the insulin-dependent glucose transporter GLUT4 in specific intracellular vesicles. These vesicles move slowly to the cell surface. Under the influence of insulin, this translocation proceeds ten times faster with significant glucose uptake as a result (Bryant et al., 2002). Through this main mechanism insulin regulates glucose homeostasis. In the brain, GLUT4 is expressed in the hippocampal formation, cortex, septum, hypothalamus and cerebellum, and in most motor and motor-associated nuclei (El Messari et al., 1998). Interestingly, the distribution of GLUT4 strongly resembles that of IRAP (Fernando et al., 2005). Furthermore, it was demonstrated that GLUT4 and IRAP are co-localised in the same cells in selected nuclei of the brain, most notably the pyramidal cells in the CA1-CA3 region of the hippocampus (Fernando et al., 2005). How the translocation occurs in the brain and whether insulin plays a key role, is not yet known. Studying this translocation and the role of IRAP might be important, because glucose was shown to have a positive effect on memory and learning in mice (Kopf et al., 2001). Additionaly, by having more IRAP at the cell surface, signal transduction by Ang IV can be studied more easily. Previous translocation studies of IRAP were hampered by a time-consuming detection of the enzyme. For example, cell surface IRAP was biotinylated and isolated by affinity chromatorgraphy, after which SDS-PAGE and western blotting was performed (Nakamura et al., 2000). Earlier indirect experiments also revealed that IRAP undergoes insulin-mediated translocation in the CHO-K1 cell model despite the fact that they do not contain GLUT4 (Johnson et al., 1998, Lim et al., 2001).

Based on our observation that CHO-K1 cells contain a large concentration of IRAP (Demaegdt et al., 2004) and on the methodology we developed to specifically detect IRAP by radiolabelled Ang IV binding, we were able to measure the insulin-regulated translocation of this protein in a more direct manner. This improved detection method relied on the inhibition of IRAP internalization by phenylarsene oxide. With this method (Demaegdt et al., submitted to Brit. J. Pharmacology),

we thus **developed, in these cells, a rapid and sensitive methodology to quantify cell-surface [^{125}I]-Ang IV binding** in the presence of phenylarsene oxide. Moreover **insulin was found to cause a rapid and transient translocation of IRAP.**

II.5. Development of novel IRAP/AT$_4$ ligands

In collaboration with Prof. T. Hallberg (University Uppsala, Sweden) and with Prof. D. Tourwe (DSCH, Vrije Universiteit Brussel), **we obtained a series of novel angiotensin IV analogues for in vitro screening with respect to their stability, potency and selectivity for human IRAP** transiently expressed in HEK293 cells. In this respect, the AT$_4$ receptor binding activity of a large series of linear Ang IV analogues were previously disclosed (Handa et al., 1999; Krishnan et al., 1999, Lee et al., 2003). However, it has now been established that these experiments only provide information about the ability of these peptides to interact with the inactive IRAP apo-enzyme (i.e. enzymes lacking the catalytic Zn^{2+}) (Lew et al,, 2003; Demaegdt et al., 2004, Laeremans et al., 2005). To obtain physiologically more relevant information about the new Ang IV analogues, we first focussed on enzyme-based assays. Accordingly, potency and IRAP versus AP-N selectivity was estimated by monitoring their ability to dose-dependently inhibit the respective aminopeptidase catalytic activities (based on the cleavage of the substrate L-leucine-p-nitroanilide). IRAP versus AT$_1$ receptor selectivity was then estimated by assessing tha ability of the peptide analogs to compete with [^{3}H]candesartan to the human AT$_1$ recepors stably expressed in CHO cells. Finally, stability of the peptide analogs towards breakdown by metalloproteases present in CHO-K1 cell membranes was estimated by comparing their competition binding curves either upon co-incubation with the radioligand [^{125}I]-Ang IV (no breakdown since the assay was performed in the presence of peptidase inhibitors) or after preincubation with the membranes in medium only. **The already obtained data are highly encouraging since both chemistry teams managed to produce peptide analogs with the aspired characteristics: i.e. high potency and selectivity for IRAP versus AP-N and especially AT$_1$ receptors, along with improved stability with respect to Ang IV.** The most successful candidates will now be used for in vivo assays.



From the Ang IV analogues obtained from M. Hallberg, it was demonstrated that shortening and modification of the C-terminal end (to yield AT4-16, see figure) as well as disulfide cyclizations of Ang IV (AT4-6, see figure) can deliver such ligands (Axén et al., 2006, 2007). Similarly, a recently synthesized analogue containing two beta amino acids obtained from Prof. Tourwe displayed similar excellent characteristics.

III. Characterisation and function of the IRAP/AT4 system *in vivo*

III.1. Introduction and validation of behavioural tests for learning and memory

To quantify the memory-promoting effects of Ang IV and several other AT_4 receptor ligands (e.g. Norleucinal, LVV-H7) within this project, the introduction of behavioural tests for memory and learning was necessary in our laboratory. In this context, **we have introduced and validated a complete set-up of the well-established 'Morris water maze' for spatial memory** 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 (Holownia and Braszko, 2003) or daily repeated i.c.v. injection of 1 nmol Nle^1-Ang IV (Olson et al., 2004) had no effect compared to the controls. However, infusion of $Nleu^1$-Ang IV (Wright et al., 1999) or daily repeated injection of $Nleu^1$-Ang IV (Pedersen et al., 1998, Olson et al., 2004) had a clear learning and memory enhancing effect in rat models for cognitive dysfunction. Since the Morris water maze task is especially valuable to assess the restoration of learning in memory impairment models, we are currently investigating the effects of continuous Ang IV administration (via an implanted osmotic pump) in the chronic amyloid-ß (1-42) model. Our preliminary data show that amyloid ß(1-42)-treated rats learned the task efficiently but were unable to develop a clear spatial strategy, whereas Ang IV and amyloid ß (1-42)-treated rats were less efficient in learning the task but demonstrated a better spatial strategy. The latter model will possibly give a lead towards the effect of Ang IV in the pathophysiology of Alzheimer's disease. We have also access to validated Barnes maze and spontaneous alternation task set-ups in collaboration with the Neuropeptides group of the Howard Florey Institute, University of Melbourne (Australia). S.Y.. Chai and A. Albiston of this group are the pioneers who discovered that the IRAP enzyme is the AT_4 binding site for Ang IV (Albiston et al., 2001).

III.2. In vivo levels of Ang IV in microdialysates

To understand the mechanism(s) by which Ang IV exerts its effects it is crucial to monitor in *in vivo* levels of this peptide in baseline conditions, following pharmacological manipulation, during a memory task, and in pathophysiological conditions. Quantifying neuropeptides in microdialysates is challenging because low concentrations are expected and thus a selective and sensitive analysis technique is required, such as nano liquid chromatography (LC) electrospray (ESI) tandem mass spectrometry (MS/MS). Other LC detection methods are inadequate to achieve the required sensitivity and selectivity. With our ESI-MS detection, we observed reduced accuracy of the LC-MS/MS method for quantifying Ang IV in dialysates due to matrix effects and instability of Ang IV. We therefore investigated the use of an internal standard (IS), i.e. a structural analogue (Norleucine1-AngIV) or a stable isotopically-labeled (SIL) analogue, to improve our method.

Linearity was improved when either of the proposed IS were applied. Only when using the SIL-IS, the repeatability of injection and the method's precision and accuracy was improved. Finally, the IS was able to correct for degradation of Ang IV in dialysates, prolonging the possible storage period of the samples. We concluded that the application of a SIL analogue is indispensable when quantifying Ang IV in dialysates using nano LC-ESI-MS/MS detection (Lanckmans et al., 2007a). We could not measure baseline levels of Ang IV although our limit of detection for Ang IV was about 10 pM (i.e. 10 attomol on column). However **we succeeded to monitor alterations in Ang IV concentrations following Ang II administration** (Lanckmans et al., 2007b).

III.3. In vivo modulation of hippocampal acetylcholine and glutamate concentrations by IRAP/AT_4 ligands

Since initial interest in Ang IV originated from its ability to increase memory acquisition and recall in several behavioural learning and memory tasks (see II.1) and since hippocampal cholinergic-glutamatergic interactions are known to be implied in learning and memory processes, we first studied whether Ang IV was able to alter cholinergic or glutamatergic hippocampal neurotransmission *in vivo*. To be able to study the effects of i.c.v. Ang IV administrations on the extracellular hippocampal acetylcholine (ACh) concentrations, we first had to introduce and profoundly validate an analytical microbore ion-pair liquid chromatography set-up with amperometric detection on a peroxidase-coated glassy-carbon electrode for the determination of ACh in brain microdialysates without the use of cholinesterase inhibitors (De Bundel et al., in preparation).

The validated method for ACh has then been applied in rat microdialysis experiments with i.c.v. administration of Ang IV and LVV-H7. We provided direct *in vivo* evidence for differences in the biological effects elicited by the AT_4 ligands, Ang IV and LVV-H7, administered in doses commonly used in rats in behavioural tasks. First, i.c.v. injection of 1 and 10 nmol Ang IV, but not LVV-H7, elicited a clear-cut drinking response. Secondly, a tendency to increase followed by a significant sustained decrease in the extracellular hippocampal ACh concentration was observed after i.c.v. administration of 1 and 10 nmol Ang IV but not of LVV-H7. Because of these conflicting data obtained with Ang IV and LVV-H7 and because of the well-established involvement of the AT_1 receptor subtype in the dipsogenic effects and central pressor effects of Ang II and its bio-active metabolites Ang III and Ang IV (de Gasparo et al., 2000), we investigated the possible role of the AT_1 receptor subtype in the effects of both ligands. In the same line, recent evidence indicated that AT_1 receptors are also involved in central pressor (Lochard et al., 2004) and peripheral vasoconstrictive effects of Ang IV (Li et al., 2006; Yang et al, in press). We showed that **i.c.v. injection of the AT_1 receptor antagonist candesartan clearly inhibited the dipsogenic effect of Ang IV as well as the Ang IV-mediated alterations in extracellular hippocampal ACh levels**. On the other hand, we were not able to provide evidence that cholinergic facilitation in the hippocampus is involved in the spatial learning and memory enhancing effect of Ang IV and LVV-H7 (De Bundel et al., in preparation). Ongoing behavioural experiments in spatial mazes with AT_1 receptor antagonists will further elucidate the role of the observed attenuation of hippocampal acetylcholine levels in the learning and memory enhancing effects of Ang IV. We further demonstrated that Ang IV was without effect on the extracellular hippocampal glutamate levels both in rats and mice, suggesting that glutamate neurotransmission is also not involved in the cognitive enhancing effects of Ang IV.

III.4. In vivo modulation of hippocampal GABA, dopamine and serotonin concentrations by Ang IV

Besides clinical evidence obtained with cholinesterase inhibitors and memantine to improve memory, 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 (Maubach, 2003). 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 (Myhrer, 2003). We therefore investigated the effects of different doses of Ang IV, administered via an i.c.v. injection or infusion, on the extracellular levels of GABA, DA and 5-HT in rats and mice. Validated microbore liquid chromatography (LC) systems running for routine analysis of monoamine and GABA dialysate concentrations are available in our laboratory. Using *in vivo* microdialysis in mice, we have preliminary data that an **i.c.v. injection of Ang IV (10 nmol/2µL) reduced the extracellular hippocampal GABA levels**. We also demonstrated that i.c.v. infusion (10 nmol/h) significantly decreased the dialysate levels of GABA obtained from rat hippocampaus (Stragier et al., 2006). It is possible that a decrease in hippocampal GABA-ergic activity is partially responsible for the memory enhancing effect displayed by Ang IV. We further showed that i.c.v. administered Ang IV (10 nmol/h) significantly elevated the extracellular DA and 5-HT concentrations in the rat hippocampus (Stragier et al., 2006). Additional 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 functioning.

III.5. Involvement of neuropeptide degradation by IRAP in the Ang IV mediated effects

Since vasopressin, somatostatin and oxytocin are known substrates for IRAP and involved in cognitive functioning (Alescio-Lautier et al., 2000, Kovacs and De Wied, 1994, Matsuoka et al., 1995), it is tempting to speculate that inhibition of IRAP's catalytic activity by Ang IV may enhance the activity of certain neuropeptide substrates. Similarly, by investigating the effects of Ang IV within the pilocarpine model for epileptic seizures, we showed that Ang IV might mediate its anticonvulsant effect by preventing IRAP-mediated degradation of somatostatin. As described above, i.c.v. administered Ang IV increased extracellular hippocampal DA and 5-HT levels. We previously showed in our laboratory that increasing the DA and 5-HT concentrations in the hippocampus is anticonvulsant in this acute pilocarpine rat seizure model (Clinckers et al., 2005). In agreement, we showed that i.c.v. administered Ang IV also protected the rats against pilocarpine-induced seizures. While the Ang IV-mediated anticonvulsant effect and increases in DA and 5-HT release were not AT_1 receptor dependent, they could be clearly blocked by concomitant i.c.v. administration of the somatostatin receptor 2 (sst2) antagonist cyanamid 154806. This suggests that Ang IV can induce sst2 receptor-mediated effects. Somatostatin-14 possesses known anticonvulsant properties (Vezzani and Hoyer, 1999) via activation of the sst2 receptor (Cammalleri et al., 2004). We demonstrated that i.c.v. administration of somatostatin-14 caused similar increases in the extracellular hippocampal DA and 5-HT levels as compared to Ang IV and also protected the rats against 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, we hypothesized that **i.c.v. administration of Ang IV caused an increase in the somatostatin-14 concentration in the brain, which then exerts its anticonvulsant effects via sst2 receptor activation**.

To check whether inhibition of IRAP indeed leads to an increase of the amount of its substrates, it would be interesting to quantify neuropeptide substrate levels in hippocampal brain dialysates following administration of Ang IV or its analogues. Due to the extreme low expected concentrations of neuropeptides in the brain (1-100 pM), this can only be achieved by using a nano LC electrospray triple quadrupole tandem mass spectrometry (MS/MS) method. As described in III.2. this method was already validated for the measurement of Ang IV, following the administration of Ang II, but was not able to detect basal extracellular Ang IV levels, showing that these levels are below the limit of detection of our method, 50 pM. (Lanckmans et al. 2007a). At the moment we are adapting our method for the quantification of the other neuropeptides such as vasopressin, somatostatin and oxytocin.

III.6. The Ang IV-induced dopamine-enhancing effect in the striatum is mediated via activation of IRAP/AT_4 and/or AP-N, acting as receptors for Ang IV

We also showed that local Ang IV administration enhances dopamine in rat striatum. Selective inhibition of AP-N, the enzyme necessary to metabolise Ang IV into smaller peptide fragments, by a compound named 7B (2(S)-benzyl-3-[hydroxyl(1'(R)-aminoethyl)-phoshynyl]propanoyl-L-tyrosine), potentiated the Ang IV-evoked dopamine release, most probably due to a lengthening of the half-life of Ang IV. Non-selective inhibition of both IRAP and AP-N by 7B alone however failed to increase dopamine levels, demonstrating that the facilitation of striatal dopamine is not mediated via inhibition of IRAP's and/or AP-N's catalytic activity. Nevertheless, since Ang IV is known to bind to both aminopeptidases, we hypothesized that **in the striatum Ang IV may act as a agonist of a receptor dimer formed by IRAP and/or AP-N** (Stragier et al., 2007).

IV. References

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V. Publication list of the EFAR research group on IRAP/AT_4 enzyme/receptor since 2004

- (1) Demaegdt H, Vanderheyden P, De Backer JP, Mosselmans S, Laeremans H, Le MT, Kersemans V, Michotte Y and Vauquelin G
  Endogenous cystinyl aminopeptidase in Chinese hamster ovary cells: characterization by [(125)I]Ang IV binding and catalytic activity.
  Biochem Pharmacol (2004) 68: 885-892. (scientific impact factor = 3.4)
- (2) Demaegdt H, Laeremans H, De Backer JP, Mosselmans S, Le MT, Kersemans V, Michotte Y, Vauquelin G and Vanderheyden P
  Synergistic modulation of cystinyl aminopeptidase by divalent cation chelators.
  Biochem Pharmacol (2004) 68: 893-900. (scientific impact factor = 3.4)
- (3) Stragier B, Sarre S, Vanderheyden P, Vauquelin G, Fournie-Zalouski MC, Ebinger G and Michotte Y
  Metabolism of angiotensin II is required for its *in vivo* effect on dopamine release in the striatum of the rat.
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  Metal ion modulation of cystinyl aminopeptidase.
  Biochem J (2005) 390: 352-357. (scientific impact factor = 4.2)
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- (7) Demaegdt H, Lenaerts PJ, Swales J., De Backer JP, Laeremans H, Le MT, Kersemans K, Vogel L, Michotte Y, Vanderheyden P and Vauquelin G
  AT4 receptor ligand interaction with cystinyl aminopeptidase and aminopeptidase N: [125I]Ang IV only binds to the cystinyl aminopeptidase apoenzyme.
  Eur. J. Pharmacol. (2006) 546: 19-27. (scientific impact factor = 2.5)
- (8) Vanderheyden P., Demaegdt H., Swales J., Lenaerts P.-J., Laeremans H., De Backer J.-P., Vogel L.K. and Vauquelin G
  Synergistic inhibition of the enzymatic activity of aminopeptidase N by divalent metal ion chelators.
  Fund. Clin. Pharmacol. (2006) 20: 613-619. (scientific impact factor = 1.7)
- (9) Axén A., Lindeberg G., Demaegdt H., Vauquelin G., Karlén A. and Hallberg M.
  Cyclic insulin-regulated aminopeptidase (IRAP)/AT4 receptor ligands.
  J. Pept. Sci. (2006) 12: 705-713. (scientific impact factor = 1.8)
- (10)Stragier B, Clinckers R, Meurs A, De Bundel D, Sarre S, Ebinger G, Michotte Y and Smolders I
  Involvement of the somatostatin 2 receptor in the anticonvulsant effect of angiotensin IV against pilocarpine-induced limbic seizures in rats.
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- (11)Stragier B, Demaegdt H., De Bundel D, Smolders I, Sarre S, Vauquelin G, Ebinger G, Michotte Y and Vanderheyden P
  Involvement of insulin-regulated aminopeptidase and/or aminopeptidase N in the angiotensin IV-induced effect on dopamine release in the striatum of the rat.
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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. Neurofibrillary tangles are made of paired helical filaments (PHFs) containing the microtubule-associated protein, tau. In Alzheimer disease, tau is hyperphosphorylated, and many serine and threonine residues, which are found to be phosphorylated in PHF tau, can be phosphorylated by GSK3 both *in vitro* and in transfected cells . Senile plaques contain an amyloid core that is mainly constituted of amyloid-β peptide (Aβ), which is derived from the amyloid precursor protein (APP). The APP gene encodes 10 different APP isoforms 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 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), 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. 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).

During the 2005-2007 period, we have studied further the metabolism of APP in different cellular models, and in particular in neurons. We have observed that lactacystin decreases Aβ peptide production by inhibiting β-secretase activity.

APP metabolism was studied in relation to a possible function of the protein. In both non amyloidogenic and amyloidogenic catabolic pathways, the cleavage of APP by γ-secretase produces the APP intracellular domain (AICD), which could be involved in the regulation of gene transcription. We have observed that Fe65 does not stabilize AICD during activation of transcription in a luciferase assay. In addition, amyloidogenic processing but not AICD production requires a precisely oriented APP dimer assembled by transmembrane GXXXG motifs.

Finally, the influence of APP phosphorylation on the processing of the protein was studied and allowed us to identity a biochemical link between the two typical lesions of Alzheimer's disease. We observed that calcium-mediated transient phosphorylation of tau and APP is followed by intraneuronal Aβ accumulation. In addition, phosphorylation of APP695 at Thr668 decreases γ-cleavage and extracellular Aβ. Finally, lithium chloride increases the production of Aβ peptide independently from its inhibition of GSK3.

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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 frontal and parietal cortical areas to the control of skilled hand movements. Although recent functional imaging studies have demonstrated that precision grasping activates a large bilateral fronto-parietal network, the actual contribution of the different areas of this network is still largely unknown. Indeed, functional brain imaging studies do not allow us to make inferences about the causal relationship between a change in the neural activity of a given region and the task at hand. In contrast, transcranial magnetic stimulation (TMS) has proved very useful to overcome this limitation by producing, in healthy subjects, a transient virtual lesion of a small brain region. Combined with a precise quantification of the deficits resulting from such virtual lesions (Duque et al., 2003), this technique permits to infer the contribution of the lesioned brain area to the task at hand. In order to determine exactly the coil position, we have used a TMS onto MRI coregistration technique developed in the laboratory (Noirhomme et al., 2004; Zosso et al., 2006) which allows us to normalize the individual coordinates of stimulation sites with respect to the Montreal Neurological Institute (MNI) brain atlas (see Davare et al., 2006, 2007).

1. Dissociating the role of ventral and dorsal premotor cortex in precision grasping

Small object manipulation and tool use are 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, including ventral (PMv) and dorsal (PMd) premotor areas. In order to dissociate the role of PMv and PMd in the control of hand and finger movements, we produced, by means of transcranial magnetic stimulation (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 a lesion of the left, contralateral, PMv disturbed the sequential recruitment of intrinsic hand muscles, all other movement parameters being unaffected. 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. Finally, an analysis of the time course of these effects allowed us to demonstrate the sequential involvement of PMv and PMd in movement preparation. 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., 2006).

2. Role of the ipsilateral primary motor cortex in controlling the timing of hand muscle recruitment

The precise contribution of the ipsilateral primary motor cortex (iM1) to hand movements remains controversial. To address this issue, we elicited transient virtual lesions of iM1 by means of transcranial magnetic stimulation (TMS) in healthy subjects performing either a grip-lift task or a step-tracking task with their right dominant hand (Davare et al., 2007). 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. The present study suggests that iM1 plays a critical role in hand movements by contributing to the processing of the muscle recruitment timing, most likely through either inhibitory or facilitatory transcallosal influences onto the contralateral M1 (cM1). It is therefore sensible to assume that iM1 contributes to shape precisely the muscular command originating from cM1.

3. Temporal dissociation of hand shaping and grip force scaling in the anterior intraparietal area (AIP) and the supplementary motor area (SMA).

In humans, both clinical and neuroimaging studies have evidenced the critical role of the posterior parietal cortex, and particularly of the anterior intraparietal area (AIP), in controlling skilled hand movements. However, the exact contribution of AIP to precision grasping remains much debated because a lot of different tasks have been shown to activate this area. To address this issue, we have used transcranial magnetic stimulation (TMS) to produce virtual lesions of the left and/or right AIP in healthy subjects performing a grip-lift task with either the right or left hand (Davare et al., 2007).

We found that virtual lesions of AIP had distinct consequences on precision grasping depending on its time of occurrence during movement preparation: lesions induced 270-220 ms before the fingers contacted the manipulandum affected specifically the hand shaping whereas lesions induced later, 170-120 ms before the contact time, altered only the grip force scaling. Another striking difference between the control of hand shaping and fingertip force by AIP is that, while a bilateral lesion was necessary to produce a deficit in hand posture, only a unilateral lesion of the left, but not right, AIP impaired the grip force scaling in either hand. These findings suggest that both the left and right AIP are involved early in movement preparation and contribute equally to the visuomotor transformations required to adjust the hand posture to the object to grasp. Then, only the left AIP is implicated in the object weight representation used to scale the grip force anticipatively.

We followed a similar approach to investigate the contribution of SMA to weight representation in the central nervous system (Schaburn et al, submitted). SMA is known to be involved in movement preparation and the generation of heavily rehearsed movement sequences. However, relatively little is known about its role in object manipulation. SMA ablation in non-human primates leads to an excessive increase in GF during object manipulation, suggesting a role for SMA in the storage of object representations. In human subjects, SMA activity has been demonstrated using fMRI

during the accurate scaling of fingertip forces. This study indicates that SMA may play a role in the anticipatory command, perhaps through recruitment of the internal model. The aim of the present study was to further examine the role of SMA in accurate GF scaling, object manipulation and internal model recruitment using rTMS induced virtual lesions of right and left SMA. We found that the left SMA plays a crucial role in internal model representation.

Altogether these two studies indicate that internal model implementation and/or storage is a distinct function of the left hemisphere.

4. Contribution of hand motor circuits to counting

The finding that number processing activates a cortical network partly overlapping that recruited for hand movements has renewed the interest in the relationship between number and finger representations. Further evidence about a possible link between fingers and numbers comes from developmental studies showing that finger movements play a crucial role in learning counting. However, increased activity in hand motor circuits during counting may unveil unspecific processes, such as shifting attention, reciting number names or matching items with a number name. To address this issue, we used transcranial magnetic stimulation to measure changes in corticospinal (CS) excitability during a counting task performed silently and using either numbers or letters of the alphabet to enumerate items (Andres et al., 2007; Andres et al., in press). We found an increased CS excitability of hand muscles during the counting task, irrespective of the use of numbers or letters, whereas it was unchanged in arm and foot muscles. Control tasks allowed us to rule out a possible influence of attention allocation or covert speech on CS excitability increase of hand muscles during counting. The present results support a specific involvement of hand motor circuits in counting since no CS changes were found in arm and foot muscles during the same task. However, the contribution of hand motor areas is not exclusively related to number processing since an increase in CS excitability was also found when letters were used to enumerate items. This finding suggests that hand motor circuits are involved whenever items have to be put in correspondence with the elements of any ordered series.

5. Central representation of hand movements

We have also 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 posterior parietal cortex (PPC) may store a repertoire of gestures tool use. Moreover, several imaging studies have suggested that, within the PPC, the critical region for hand movement representations may be the left inferior parietal lobule (see Fadiga et al., 2005 for review). To address this issue experimentally, we have compared, in healthy subjects, the consequences of virtual lesions of Brodmann area 7 (BA7) and of the supramarginal gyrus (SMG) on a hand rotation task and on a control task using letters. We have found that only a virtual lesion of the SMG, as induced with TMS, affected the reaction time in the hand rotation task performance was only altered by a BA7 lesion (Pelgrims et al., under revision). We have also investigated the role of the primary motor cortex into motor imagery (Pelgrims et al., submitted).

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

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. 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 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.

We also investigated 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 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. 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. The glucocorticoid-induced receptor (GIR)

Glucocorticoid-induced receptor (GIR) is an orphan GPCR with predominant expression in brain and thymus. High levels of GIR expression have been described in limbic forebrain and hypothalamic

regions of the brain of mouse, rat and human, suggesting a role for GIR in memory, cognition, stress, reward or the control of emotion. We have generated a knock-out model for GIR in which part of the coding region is replaced by a tau-LacZ reporter gene. Using this reporter gene, we confirmed the high expression of GIR in scattered large striatal neurons coexpressing choline acetyl-transferase, a specific marker for cholinergic neurons. Strong labelling was also observed in neurons of the olfactory bulb, the olfactory tubercle, the thalamus, and less abundantly in the piriform cortex and hippocampus. The knockout mice were tested in a number of behavioural settings. The mice displayed hyperlocomotion in the open field and were prone to anxiety. Motor coordination was affected, particularly in old mice, as shown in the rotarod and strength grip tests. In situ hybridization has shown reduced expression of the proenkephalin gene and overexpression of substance P and prodynorphin genes, suggesting increased activity of dopamine D_1-expressing neurons and reduced activity of D2-expressing neurons, while the number of D_1 and D_2 binding sites in the striatum appeared unchanged. Microarray analysis of the striatum has revealed overexpression in knockout mice of a number of genes also upregulated following acute or chronic cocaine treatment (c-fos, egr1, egr2, PP-1, Na/K ATPase). Cocaine administration resulted in stimulated locomotion in both genotypes, but more efficiently in KO animals. The treatment with D_1 and D_2 agonists or antagonists also affected the KO and wild-type mice differently. The mechanisms underlying the apparent hyperactivity of the dopaminergic system in the striatum of GIR knockout mice are still being investigated (Laurent et al. unpublished).

4. 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).

5. Characterization of the FPRL2 receptor

We have reported the identification of the peptide F2L, a highly conserved acetylated peptide derived from the amino-terminal cleavage of heme-binding protein, as a potent chemoattractant for human monocytes and dendritic cells (Migeotte et al. 2005). F2L inhibits LPS-induced human dendritic cell maturation, and is able to activate the human receptors FPRL-1 and FPRL2, two members of the FPR family, with highest selectivity and affinity for FPRL2. To facilitate delineation of mechanisms of F2L action in vivo, we have attempted to define its mouse receptors. This is complicated by the nonequivalence of the human and mouse FPR gene families (three vs at least eight members, respectively). When cell lines were transfected with plasmids encoding the eight mouse receptors, only the one expressing the receptor Fpr2 responded to F2L (EC_{50} approximately

400 nM for both human and mouse F2L in both calcium flux and cAMP inhibition assays). This value is similar to F2L potency at human FPRL1. Consistent with this, mouse neutrophils, which like macrophages and dendritic cells express Fpr2, responded to human and mouse F2L in both calcium flux and chemotaxis assays with EC_{50} values similar to those found for Fpr2-expressing cell lines (approximately 500 nM). Moreover, neutrophils from mice genetically deficient in Fpr2 failed to respond to F2L. Thus, Fpr2 is a mouse receptor for F2L, and can be targeted for the study of F2L action in mouse models (Gao et al. 2007).

6. 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. $ChemR23^+$ DCs were observed in secondary lymphoid organs (lymph nodes and tonsils), as well as in dermis from normal skin, whereas Langerhans cells were negative. Chemerin expression was detected on high endothelial venules in secondary lymphoid organs and in dermal endothelial vessels of lupus erythematosus skin lesions. 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). We also showed that blood $CD56^{low}CD16^+$ natural killer (NK) cells selectively express functional ChemR23. Biopsies obtained from patients with oral lichen planus presented an infiltration of NK cells expressing ChemR23, as well as myeloid and plasmacytoid $ChemR23^+$ dendritic cells. These findings suggest a role for the ChemR23/chemerin axis in the recruitment of blood NK cells and in the colocalization of NK cells and DC subsets in pathologic peripheral tissues (Parolini et al. 2007).

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).

Chemerin and ChemR23 were also shown to be secreted by adipose tissue and to influence adipose cell function. Their expression levels were up-regulated in mice fed a high-fat diet. Both chemerin and chemerinR mRNA expression increased during the differentiation of 3T3-L1 cells and human preadipocytes into adipocytes. Recombinant chemerin induced the phosphorylation of ERK1/2 and lipolysis in differentiated 3T3-L1 adipocytes. Thus, chemerin might behave as an adipokine regulating adipocyte function by autocrine/paracrine mechanisms (Roh et al. 2007).

7. 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, particularly in the contexts of addition and pain.

Caffeine has biphasic effects on locomotion, and blockade of the adenosine A_{2A} receptor 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 investigated whether the appetitive properties of caffeine can be linked to the blockade of A_{2A} receptors, by comparing knockout and wild-type mice in a caffeine intake paradigm. When mice had ad libitum access to caffeine and water in a two-bottle paradigm, KO mice drank less caffeinated solution, demonstrating a reduced appetite for caffeine as compared to WT mice, revealing an important role for the A_{2A} receptor in the appetitive properties of caffeine (El Yacoubi et al. 2005).

A_{2A} adenosine and D_2 dopamine receptors are colocalized in the same neurons in discrete brain areas, and the dopaminergic transmission plays a crucial role in the addictive properties of drugs of abuse, such as cocaine. We have investigated the specific role of A_{2A} adenosine receptors in cocaine-induced behavioral responses related to its addictive properties. Acute cocaine induced a similar increase of locomotor activity in mice lacking A_{2A} adenosine receptors and wild-type littermates. Cocaine-induced locomotor sensitization and conditioned place preference were also maintained in A_{2A} knockout mice. Nevertheless, knockout mice showed a lower rate of cocaine self-administration than wild-type mice in fixed ratio schedules of reinforcement. Moreover, a reduction in the maximal effort to obtain a cocaine infusion was found in A_{2A} knockout mice under a progressive ratio schedule. In addition, a vertical shift of the cocaine dose-response curve was observed in mice lacking A_{2A} receptors. These results demonstrate that A_{2A} adenosine receptors play an important role in cocaine addictive properties, and that these receptors are required to develop the addictive effects of this drug (Soria et al. 2006).

The non-selective A_{2A} antagonist caffeine has also been reported to modify nicotine-induced locomotor and reinforcing effects. We investigated the specific role of A_{2A} receptors in the behavioural responses induced by nicotine. Acute nicotine administration induced a similar decrease

of locomotor activity in A_{2A} knockout mice and wild-type littermates. Acute antinociceptive responses elicited by nicotine in the tail-immersion and hot-plate tests were unaffected in mutant mice. The rewarding properties of nicotine were investigated using the place-conditioning paradigm. Nicotine-induced conditioned place preference was suppressed in A_{2A} knockout mice. Accordingly, in vivo microdialysis studies revealed that the extracellular levels of dopamine in the nucleus accumbens were not increased after nicotine administration in mutant mice. Finally, the administration of the nicotinic antagonist mecamylamine in nicotine-dependent mice precipitated a similar withdrawal syndrome in both genotypes. Together, these results identify A_{2A} adenosine receptors as an important factor that contributes to the rewarding properties of nicotine (Castane et al. 2006).

Dopamine and adenosine receptors share a considerable overlap in their regional distribution, especially in the basal ganglia, and relationships between them (both antagonistic and synergistic) have been described. We investigated dopaminergic and purinergic systems in mice with ablations of individual dopamine or adenosine receptors. In situ hybridization histochemistry and autoradiography was used to examine the level of mRNA and protein expression of specific receptors and transporters in dopaminergic pathways. Expression of the mRNA encoding the dopamine D_2 receptor was elevated in the caudate putamen of D_1, D_3 and A_{2A} receptor knockout mice; this was mirrored by an increase in D_2 receptor protein in D_1 and D_3 receptor knockout mice, but not in A_{2A} knockout mice. Dopamine D_1 receptor binding was decreased in the caudate putamen, nucleus accumbens, olfactory tubercle and ventral pallidum of D_2 receptor knockout mice. In substantia nigra pars compacta, dopamine transporter mRNA expression was dramatically decreased in D_3 receptor knockout mice, but elevated in A_{2A} receptor knockout mice. All dopamine receptor knockout mice examined exhibited increased A_{2A} receptor binding in the caudate putamen, nucleus accumbens and olfactory tubercle. These data are consistent with the existence of functional interactions between dopaminergic and purinergic systems in these reward and motor-related brain regions (Short et al. 2006).

We also investigated the involvement of adenosine receptors in the interaction between paracetamol and caffeine in mice, using the adenosine A_{2A} receptor antagonist SCH58261 and the adenosine A_{2B} receptor antagonist PSB1115, in the tail immersion and hot-plate tests. Paracetamol was antinociceptive in both tests, but, in contrast to previous studies, caffeine was pronociceptive in the tail immersion test, and reduced the effects of paracetamol in both tests. SCH58261 was antinociceptive in both tests and in its presence paracetamol had no further effect. PSB1115 had little effect alone but potentiated the effect of paracetamol in the hot-plate test and abolished it in the tail immersion test. These results suggest that adenosine A_{2B} receptors may be involved in the action of paracetamol in a pathway-dependent manner, and also support the existence of pronociceptive adenosine A_{2A} receptors (Godfrey et al. 2006).

Mice lacking the adenosine A_{2A} receptor are hypoalgesic, and previous studies have suggested a role for the A_{2A} receptor in sensitizing afferent fibres projecting to the spinal cord. To test this hypothesis, formalin was injected into the paw and nociceptive responses were measured in wildtype and A_{2A} receptor knockout mice. There was a significant reduction in nociception

associated with sensory nerve activation in the knockout mice as measured by time spent biting/ licking the formalin-injected paw and number of flinches seen during the first phase, but only the number of flinches was reduced during the second inflammatory phase. In addition, the selective adenosine A_{2A} antagonist SCH58261 also antagonised both phases of the formalin test. [^{3}H]-Substance P binding to NK_1 receptors was unaltered but there was a substantial reduction in binding of [^{3}H]-MK801 to NMDA glutamate receptors in all regions of the spinal cord from knockout mice. The decrease in NMDA glutamate receptor binding may reflect reduced peripheral sensory input to the spinal cord during development and could relate to the hypoalgesia in this genotype. These results support a key role for the adenosine A_{2A} receptor in peripheral nociceptive pathways (Hussey et al. 2007).

8. 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. The functional interaction between endogenous opioid and cannabinoid receptor systems was tested in the caudate putamen and nucleus accumbens. We examined by autoradiography the functional activity and density of μ-, κ- and δ-opioid receptors in both brain regions of CB_1 knockout mice. [^{35}S]GTPγS binding 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 the three receptors between mutant and wild-type mice. No differences were found in densities or activities of opioid receptors in the nucleus accumbens. Deletion of CB_1 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 (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. CB_1 receptor KO and WT mice were trained to intravenously self-administer cocaine. Only 25% of KO vs 75% of WT littermates acquired a reliable operant responding to self-administer cocaine, 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. These results demonstrate 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).

A single administration of cocaine or D-amphetamine produces acute hyperlocomotion and long-lasting increased sensitivity to subsequent injections. We investigated the role of the CB_1 receptor in these effects. The acute locomotor response to cocaine was normal in mice pretreated with the CB_1 inverse agonist AM251, whereas no sensitization was observed in response to a second administration a week later. Locomotor responses to cocaine and D-amphetamine were decreased in CB_1 KO mice, and sensitization was impaired. To determine how CB_1 controls long-lasting effects of psychostimulants, we studied cocaine-activated signaling pathways. Cocaine-induced cAMP-dependent phosphorylation of glutamate receptor 1 was altered in the striatum of CB_1 KO mice but not of AM251-treated mice. In contrast, cocaine-induced phosphorylation of ERK was blocked in both CB_1 KO and antagonist-pretreated mice. Conditional deletion of CB_1 in forebrain principal neurons or GABAergic neurons prevented cocaine-induced ERK activation in dorsal striatum and nucleus accumbens. The endocannabinoid system thus regulates neuronal circuits critical for long-lasting effects of cocaine, presumably by acting on CB_1 receptors located on terminals of striatal medium spiny neurons (Corbillé et al. 2007).

We investigated the neuroadaptations induced by chronic alcohol exposure on both NMDA and $GABA_A$ receptors in CB_1 KO mice. Basal levels of hippocampal [^{3}H]MK-801 binding sites were decreased in CB_1 KO mice and these mice were also less sensitive to the locomotor effects of MK-801. Basal level of both hippocampal and cerebellar [^{3}H]muscimol binding was lower and sensitivity to the hypothermic effects of diazepam and pentobarbital was increased in CB_1 KO mice. Transcript levels for $GABA_A$ and NMDA receptor subunits were lower in striatum of CB_1 KO mice. [^{3}H]MK-801 binding sites were increased in cerebral cortex and hippocampus after chronic ethanol ingestion only in wild-type mice. Chronic ethanol ingestion reduced the number of [^{3}H]muscimol binding sites in cerebral cortex, but not in cerebellum, only in WT mice. We conclude that lifelong deletion of CB_1 receptors impairs neuroadaptations of both NMDA and $GABA_A$ receptors after chronic ethanol exposure and that the endocannabinoid/CB_1 receptor system is involved in alcohol dependence (Warnault et al. 2007).

We studied the effects of cannabinoids on contextual conditioned fear responses. CB_1 KO and WT mice were exposed to a brief session of electric shocks, and their behavior was studied in the same context 24 h later. In wild-type mice, shock exposure increased freezing and resting, and decreased locomotion and exploration. The genetic disruption of the CB_1 receptor abolished the conditioned fear response. The CB1 antagonist AM-251 reduced the peak of the conditioned

fear response when applied 30 min before behavioral testing in wild-type mice. The cannabinoid agonist WIN-55,212-2 markedly increased the conditioned fear response in wild-type mice, the effect of which was potently antagonized by AM-251. Thus, cannabinoid receptor activation appears to strongly promote the expression of contextual conditioned fear in this setting (Mikics et al. 2006).

The CB_1 receptor has been implicated in the inhibitory control of learning and memory. We compared the behavioral response of CB_1 KO mice with animals receiving the CB_1 inverse agonist SR141716A (rimonabant) in terms of acquisition and retention of a habituation task and changes in cerebral monoamines. The acute and chronic invalidation of the CB_1 receptor resulted in an increase of behavioral habituation during the first exposure to an open field, indicative of enhanced acquisition of the task. CB_1 KO mice, but not SR141716A-treated animals, showed enhanced retention of the habituation task when re-tested 48 h and 1 week subsequent to the first exposure to the open field, respectively. The facilitation of retention of the habituation task in CB_1 KO mice was accompanied by a selective and site-specific increase in serotonin activity in hippocampus and SR141716A-treated animals displayed antidepressant-like neurochemical alterations of cerebral monoamines (most parameters of monoaminergic activity were increased especially in dorsal striatum and hippocampus). Taken together, these findings demonstrate that the genetic disruption of the CB_1 receptor gene can cause an improvement of behavioral habituation, which is considered to represent a form of 'non-associative' learning. Furthermore, our data support the existence of a SR141716A-sensitive site that is different from the classical CB_1 receptor (Thiemann et al. 2007). We also investigated the possible interactions between the cannabinoid and cholinergic systems in memory and learning processes in two behavioural models, the active avoidance and the object recognition test. Nicotine did not modify the performance of CB_1 knockout and wild-type mice in the active avoidance paradigm, whereas scopolamine impaired the performance in both genotypes. Physostigmine increased the active avoidance performance in wild-type but not in CB_1 knockout mice. The CB_1 antagonist Rimonabant did not modify the performance in the active avoidance test, given alone or co-administered with nicotine. In contrast, nicotine enhanced the performance in the object recognition task but this response was attenuated by rimonabant co-administration. These findings revealed that the cognitive effects of nicotine and physostigmine were attenuated in the absence of CB_1 receptor activity, while scopolamine effects were independent from CB_1 receptors (Bura et al. 2007).

Endocannabinoids play central roles in retrograde signaling at a wide variety of synapses throughout the CNS. We showed that principal cell populations of the hippocampus express high levels of diacylglycerol lipase alpha (DGL-alpha), the enzyme involved in generation of the endocannabinoid 2-arachidonoyl-glycerol (2-AG). Immunostaining revealed that this lipase was concentrated in heads of dendritic spines throughout the hippocampal formation and highly compartmentalized into a wide perisynaptic annulus around the postsynaptic density of axospinous contacts. On the opposite side of the synapse, the axon terminals forming these excitatory contacts were found to be equipped with presynaptic CB_1 cannabinoid receptors. This precise anatomical positioning suggests that 2-AG produced by DGL-alpha on spine heads may be involved in retrograde synaptic signaling at glutamatergic synapses, whereas CB_1 receptors located on the afferent terminals are

in an ideal position to bind 2-AG and thereby adjust presynaptic glutamate release as a function of postsynaptic activity. We propose that this molecular composition of the endocannabinoid system may be a general feature of most glutamatergic synapses throughout the brain and may contribute to homosynaptic plasticity of excitatory synapses and to heterosynaptic plasticity between excitatory and inhibitory contacts (Katona et al. 2006).

Cannabinoids protect the brain against ischemia and related forms of injury, and stimulate neurogenesis in the adult brain. We investigated whether cannabinoid-induced neurogenesis, like cannabinoid neuroprotection, is mediated through alterations in NO production. We measured neurogenesis in dentate gyrus (DG) and subventricular zone (SVZ) of CB_1 KO and wild-type mice, some of whom were treated with the cannabinoid agonist Win 55212-2 or the NO synthase inhibitor 7-nitroindazole (7-NI). NOS activity was increased by approximately 25%, whereas bromodeoxyuridine (BrdU) labeling of newborn cells in DG and SVZ was reduced by approximately 50% in CB_1-KO compared with wild-type mice. 7-NI increased BrdU labeling in both DG and SVZ and to a greater extent in CB_1-KO than in wild-type mice. In addition, Win 55212-2 and 7-NI enhanced BrdU incorporation into neuron-enriched cerebral cortical cultures to a similar maximal extent and in nonadditive fashion, consistent with a shared mechanism of action. Double-label confocal microscopy showed coexpression of BrdU and the neuronal lineage marker doublecortin (Dcx) in DG and SVZ of untreated and 7-NI-treated CB_1-KO mice, and 7-NI increased the number of Dcx- and BrdU/Dcx-immunoreactive cells in SVZ and DG. Thus, cannabinoids appear to stimulate adult neurogenesis by opposing the antineurogenic effect of NO (Kim et al. 2006).

Neuropathic pain is a clinical manifestation characterized by the presence of spontaneous pain, allodynia and hyperalgesia. The involvement of CB_1 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 and WT mice, 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).

The cannabinoid system is immunomodulatory and has been targeted as a treatment for multiple sclerosis. Using the mouse model of experimental autoimmune encephalomyelitis (EAE), we investigated the role of the CB_1 and CB_2 receptors in regulating CNS autoimmunity. We found that CB_1 receptor expression by neurons, but not T cells, was required for cannabinoid-mediated EAE suppression. In contrast, CB_2 receptor expression by encephalitogenic T cells was critical for controlling inflammation associated with EAE. CB_2-deficient T cells in the CNS during EAE exhibited reduced levels of apoptosis, a higher rate of proliferation and increased production of

inflammatory cytokines, resulting in severe clinical disease. Together, our results demonstrate that the cannabinoid system within the CNS plays a critical role in regulating autoimmune inflammation, with the CNS directly suppressing T-cell effector function via the CB_2 receptor (Maresz et al. 2007).

9. 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 G_s 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).

10. Huntington disease model

There is presently a controversy in the litterature regarding the effects of minocycline in the R6/2 transgenic model of Huntington's disease. We have therefore tested this tetracycline in another model, the N171-82Q strain. Ten miligrams per kilogram minocycline was given daily from the age of 2 mo, corresponding to an early symptomatic stage of Huntington's disease. We did not observe improvement in survival, weight loss, or motor function in treated transgenic mice. In addition, minocycline failed to mitigate the ventricle enlargement as well as the striatal and cortical atrophies observed in the transgenic line. Using high-performance liquid chromatography, it was observed that minocycline was similarly present in the plasma and the brain of both wild-type and N171-82Q mice following 14 daily injections. Using Western blot, we showed that the increased expression of procaspase-1 induced by the transgene in the cortex was significantly reduced by the antibiotic. These data support that despite minocycline crossing the blood-brain barrier and inhibiting procaspase-1 expression, it did not provide protection in this Huntington's disease model. The present data do not support minocycline as a beneficial drug for Huntington's disease (Mievis et al. 2007).

We have also evaluated the neuroprotective effects of citicoline in phenotypic models of Huntington's disease induced by either the mitochondrial inhibitor 3-nitropropionic acid or the N-methyl-D-aspartate agonist quinolinic acid, which reproduce respectively the metabolic defect or the excitotoxicity seen in the disease. We found that citicoline failed to reverse behavioural

and histological alterations induced by both neurotoxins. In addition, citicoline did not reduce PC12 cell death induced by the expression of an N-terminal fragment of mutated Huntingtin. Altogether, our results suggest that citicoline is not a potential therapeutic agent for the treatment of Huntington's disease (Mievis et al. 2007).

11. Chemokine receptors

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). In G protein-coupled receptors (GPCRs), the interaction between the cytosolic ends of transmembrane helix 3 (TM3) and TM6 was shown to play an important role in the transition from inactive to active states. According to the currently prevailing model, the arginine of the conserved DRY motif located at the cytosolic end of TM3 interacts with acidic residues in TM3 and TM6 at the resting state and shift out of this polar pocket upon agonist stimulation. However, the acidic residue in TM6 is not conserved in chemokine receptors. Mutagenesis of the relevant residies and functional testing indicate that the constitutive and ligand-promoted activity of CCR5 can be modified by modulating the interaction between the DRY motif in TM3 and residues in TM6. Therefore, the overall structure and activation mechanism appear to be well conserved in GPCRs, while the molecular interactions locking the inactive state must be different in different classes of receptors (Springael et al. 2007). The three-dimensional model of CCR5 was also used to screen in silico a library of 1.6 million commercially available compounds. A hit list of 59 compounds was obtained, out of which 10 exhibited a detectable binding activity to CCR5. Most binders were shown to be agonists of the receptor. One of the agonists was shown to promote efficient receptor internalization, which is a process therapeutically favorable for protection against HIV-1 infection (Kellenberger et al. 2007).

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). In a mouse model of acute pancreatitis (promoted by ip injection of cerulein), lack of CCR5 exacerbated pancreatitis and led to increased levels of CC chemokines and a more pronounced pancreatic inflammatory infiltrate. These results suggest that CCR5-expressing cells recruited to pancreas modulate the severity of acute pancreatitis (Moreno et al. 2006).

The regulation by various cytokines of CXCL10 expression was studied in human fibroblasts and microvascular endothelial cells. IL-1β and TNF-α synergized with IFN-α,β or γ for CXCL10 induction. High synovial CXCL10 concentrations were found in autoimmune arthritis (ankylosing spondylitis, psoriatic arthritis and rheumatoid arthritis) (Proost et al. 2006).

Human T-cell leukemia virus type-1 (HTLV-1) is associated with adult T-cell leukemia (ATL) and neurological syndromes. HTLV-1 encodes the oncoprotein Tax-1 that modulates viral and cellular

gene expression leading to T-cell transformation. We reported an interaction between HTLV-I Tax oncoprotein and the G protein beta subunit. The G protein beta subunit inhibits Tax-mediated viral transcription, while Tax-1 perturbs G protein beta subcellular localization. Functional evidence for these observations was obtained using conditional Tax-1-expressing transformed T-lymphocytes, where Tax expression correlated with activation of the SDF-1/CXCR4 axis. HTLV-1 has therefore developed a strategy based on the activation of the SDF-1/CXCR4 axis in the infected cell (Twizere et al. 2007).

Chemokines and their receptors play important roles in various aspects of tumoral processes, including in determining the metastatic destination of tumor cells. We analyzed in vitro and in vivo, how CCR6 expression could alter the behavior of Lewis lung carcinoma (LLC) cells, which were shown to express low levels of the CCR6 ligand, CCL20 (LARC). The expression of CCR6 decreased the number of metastases without affecting the tumor-forming ability of LLC cells. This was correlated with a decrease in clonogenicity in agar, and increased adhesion to type-IV collagen. Thus, expression of CCR6 in tumor cells, associated with the local production of CCL20, decreased the metastatic potential of the LLC line. We propose a model, in which the expression of a chemokine receptor in tumor cells can act as a metastasis-suppressor, or a metastasis-promoting factor, according to the expression, or the absence of expression of the cognate ligand(s) in the tumor (Sutherland et al. 2007).

12. P2Y receptors

Extracellular ATP is known to affect the maturation of monocyte-derived dendritic cells mainly by regulation of cytokines and costimulatory molecules. We have shown that adenine nucleotides inhibit the release of MCP-1 (CCL2) and MIP-1α (CCL3) by human monocyte-derived dendritic cells through the P2Y11 and P2Y1 purinergic receptors, resulting in a strongly reduced capacity to attract other leucocyte populations (Horckmans et al. 2006).

13. Dimerization of GPCRs and functional consequences

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 (El Asmar et al. 2005, Springael et al. 2005). We have further provided evidence from bioluminescence resonance energy transfer experiments that stimulation by chemokines does not influence the CCR2/CCR5 heterodimerization status. In addition, we showed that the rate of radioligand dissociation from one unit of the heterodimer in "infinite" tracer dilution conditions is strongly increased in the presence of an unlabeled chemokine ligand of the other unit. These results demonstrate unambiguously that the interaction between heterodimer units is of allosteric nature. Agonists, but also some monoclonal antibodies, could promote such negative binding cooperativity, indicating that this phenomenon does not require the full conformational change associated with receptor activation. Finally, we show that G protein coupling is required for high-affinity binding of MIP-1β (CCL4) to CCR5 and that the dissociation from G proteins, after incubation

with Gpp(NH)p, promotes the release of prebound radiolabeled chemokines with kinetics similar to those measured after the addition of an excess of unlabeled chemokines. These observations suggest that the association with G proteins probably participates in the negative cooperativity observed between receptor monomers (Springael et al. 2006).

We have extended these observations to heterodimers formed by CCR2 and CXCR4, which are more distantly related. We also show that specific antagonists of one receptor inhibit the binding of chemokines to the other receptor as a consequence of their heterodimerization, both in recombinant cell lines and primary leukocytes. This resulted in a significant functional cross-inhibition in terms of calcium mobilization and chemotaxis. These data demonstrate that chemokine receptor antagonists regulate allosterically the functional properties of receptors on which they do not bind directly, with important implications on the effects of these potential therapeutic agents (Sohy et al. 2007).

Such negative cooperativity within homo- and heterodimers can likely be extended to other classes of G protein-coupled receptors, including in the central nervous system. Allosteric regulation within di(oligo)mers thus implies that the pharmacological properties of a given receptor subtype can be influenced by the array of dimerization partners coexpressed in each particular cell type. Ligands could thus act as agonists or antagonists on one receptor, while modulating allosterically the function of a variety of other receptors to which they do not bind directly. Allosteric regulation across GPCR oligomeric interfaces will have major implications in the pharmacology of these receptors in vivo and in the physiopathology of the diseases in which they are associated. It will likely affect the design of drug discovery programs, which rely mostly on the overexpression of the receptor of interest in a cell line, thereby focusing on homo-oligomers and ignoring the potential effects of other partners (Springael et al. 2007, Pin et al. 2007). As part of a consortium, we have proposed the requirements for an information system that can manage the elements of information needed to describe comprehensively the phenomena of both homo- and hetero-oligomerization of GPCRs (Skrabanek et al. 2007).

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Long-term memory in mouse hippocampal slices

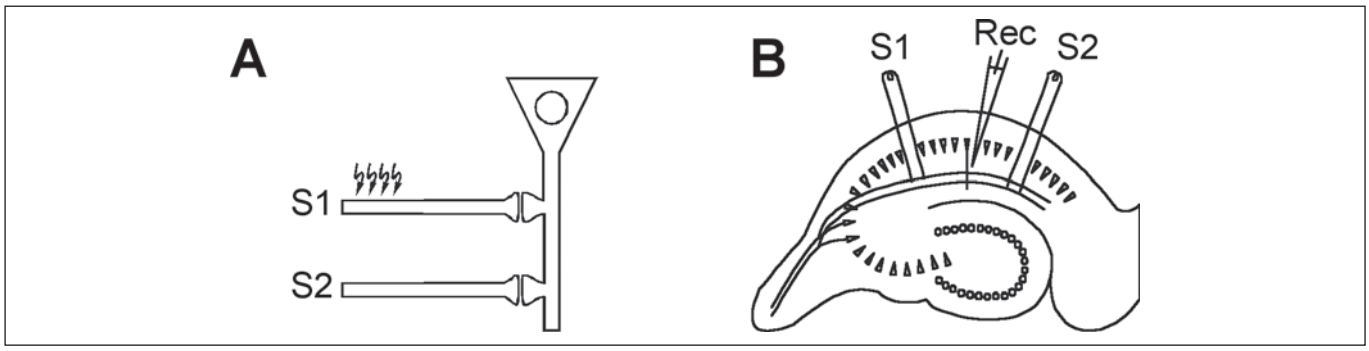
Background

For neurocomputing theorists, every memory is encoded in a neuronal network thanks to a change in the distribution of its synaptic weights. Among the possible biological mechanisms, long-term potentiation (LTP) is the favored candidate to be at the basis of memory storage because this activity-induced increase in synaptic strength is (1) durable and (2) input-selective (restricted to the activated synapses). This phenomenon can be studied on an *in vitro* preparation. In a thin hippocampal slice (0.5 mm thick) maintained artificially alive it is possible to induce an LTP of the strength of the synapses by appropriate electrical stimulation of the presynaptic fibers. Two temporal phases of LTP can be induced and explored. A short-lasting LTP (S-LTP), which lasts 1-2 h, can be triggered by application of a single train of high frequency stimulation (HFS). S-LTP has been intensively studied and the underlying mechanisms are relatively well known. They consist of incorporation of extra chemoreceptors in the postsynaptic membrane and phosphorylation of proteins. A more durable LTP can also be elicited in hippocampal slices. This long-lasting LTP (L-LTP) is known for long but the mechanisms underlying its late phase are very poorly understood. Classically, L-LTP has three attributes. The first is of course its "lateness", the second is the fact that its induction necessitates multiple trains of tetanic stimuli and the third is that its induction requires a change in gene expression and protein synthesis.

During these three last years, our group tackled several phenomena related yo the development of L-LTP in hippocampal slices.

1. Age and synaptic specificity of L-LTP.

Memory shows age-related decline. According to the current prevailing theoretical model, encoding of memories relies on modifications in the strength of the synapses connecting the different cells within a neuronal network. The selective increases in synaptic weight are thought to be biologically implemented by long-term potentiation. During this year, we explored the possibility that input specificity of LTP could vanish with aging and hence hinder memory.



Two groups of mice (2 and 12 months old) were compared. Two distinct bundles of Schaffer collaterals were stimulated (S1 and S2) and the responses that both of them evoked in a same group of CA1 pyramidal neurons were recorded. In young mice, we confirmed the well-established

input selectivity of LTP. When 4 trains were applied through S1, an LTP developed in the synapses tested via S1, while the strength of the synapses tested via S2 remained unchanged. This situation was totally different in mice aged 12 months. In this group of mice, application of 4 trains of stimulation through S1 triggered not only an LTP in the synapses tested via S1, but also a progressively developing increase in the strength of the synapses tested via S2. We further demonstrated that this phenomenon was suppressed by blocking either L-type Ca^{++} channels or Ca^{++}-induced Ca^{++} release, both well known to become dysregulated with aging.

Our results not only demonstrate that synapse specificity of LTP breaks down with aging but also strongly suggest that the induction of the formation of a "tag" by synaptic activity is mediated by Ca^{++}. (Ris and Godaux, Learning and Memory, 2007)

2. Sexual dimorphisms in the cascades involved in L-LTP

There are several signaling cascades allowing to the Ca^{++} entry to trigger protein synthesis. At least three signaling cascades can make this. First cascade. Ca^{++} activates adenylate cyclase. The resulting increase in AMPc concentration stimulates protein kinase A (PKA). As a result, PKA dissociates into its two catalytic domains and its two regulatory domains. The dissociated catalytic domain of PKA translocates into the nucleus and via CREB phosphorylation, triggers protein synthesis. A second cascade involved mitogen-activated protein kinase (MAPK). Ca^{++} activates MAPK-KK (kinase of kinase of kinase) which, in turn, activates MAPK-K which, in turn, phosphorylates MAPK. Phosphorylated MAPK translocates into the nucleus and also via CREB phosphorylation, triggers protein synthesis. The third cascade involves CaMKIV (calcium calmodulin kinase number four), an enzyme constitutionally located in the nucleus. This enzyme, whose activity leads to protein synthesis, is activated by Ca^{++} associated with calmodulin.

Surprisingly, we found that male and female mammals did not use the same signaling cascades.

CaMKIV is one of the two main targets of CaMKK (kinase of members of the CaMK family) which has two isoforms (CaMKKα and CaMKKβ). In collaboration with Prof. Giese at the University College of London who made CaMKK (α or β) knocked out mice, we explored whether CaMKK (α and β) played a role in the late phase of L-LTP. Giese's team found deficits in certain types of learning which were different in male and female mice. Consequently, we tested slices from female mice and male mice separately. We found no defect of L-LTP both in the male and in the female CaMKKα hippomorphic mice. (Mizuno et al. Mol Cell Biol 2006).

By contrast, we found that CaMKKβ had a male-specific role in L-LTP. The late phase of L-LTP was impaired in slices from male CaMKKβ knocked out mice but not in female mutants.(Mizuno et al. Neuroscience 2007).

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.(Ris et al. Eur J Neurosci. 2005)

3. Metaplasticity of L-LTP

In fact, the capability of a synapse to change its strength as a function of its immediately previous activity (synaptic plasticity) is also dependent of the story of the synapse. This phenomenon is known as metaplasticity. We discovered two new types of metaplasticity.

a) In area CA1 of hippocampal slices which are allowed to recover from slicing "in interface" and where recordings are carried out ininteface, a single 1-sec train of 100-Hz stimulation triggers a short-lasting long-term potentiation (S-LTP),which lasts 1-2h, whereas multiple 1-sec trains induce a long-lasting LTP (L-LTP), which lasts several hours. Where all recordings were performed in interface, we found that allowing the slices to recover "in submersion" had dramatic metaplastic effects. In these conditions, a single 1-sec train at 100 Hz induced an L-LTP which lasted at least 4 h and was dependent on protein synthesis. (Capron et al. Learning and Memory 2006)

b) On a preparation where recovery and recordings occurred "in interface", we found that when a single train of HFS was applied 45 min after application of the SK channel blocker apamin, it induced an L-LTP of several hours, instead of an S-LTP of 1-2 h. We found that this metaplastic effect of apamin was crucially dependent on the NO-synthase pathway. Ris et al. Learning and Memory 2007)

4. L-LTP and Alzheimer's Disease

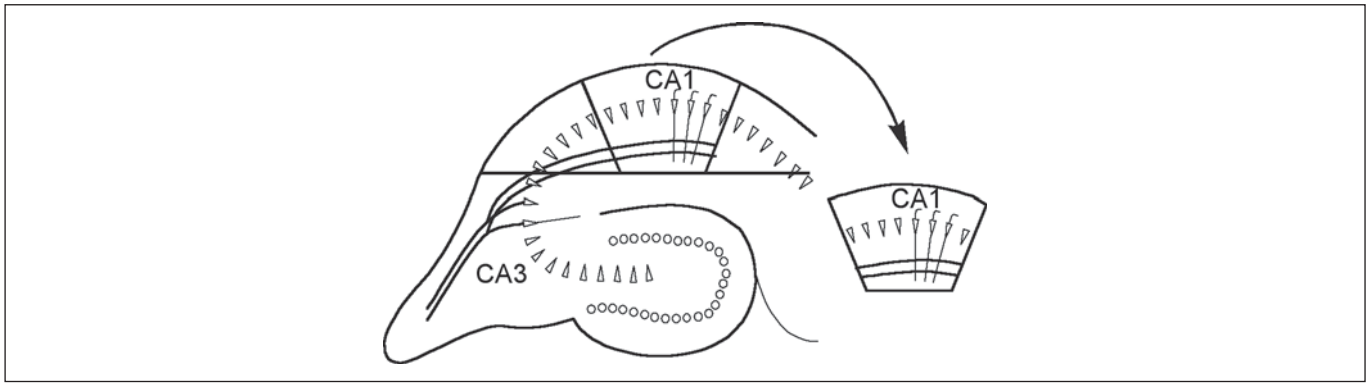
Alzheimer's Disease is characterized by a devastating deterioration of cognitive functions and memory. Mutations in presenilin and in the amyloid precursor protein (APP) are the most common cause of early onset cases of familial Alzheimer's disease. As L-LTP is believed to be at the basis of memory, it is important for understanding the fundamental mechnanisms of L-LTP to try to identify the processes impaired in AD. On this topic, we collaborate with the laboratory of Prof. Van Leuven (KUL) which has generated different strains of transgenic mice in relation with AD.

We also demonstrated that PS1(n-/-) displayed a subtle impairment in Schaffer collateral hippocampal LTP as opposed to normal LTP in wild-type PS1 mice, and a facilitated LTP in mutant PS1[A246E] mice (Dewachter et al. Neurobiol Aging. 2007, Jan 11).

We recently demonstrated that both NMDA-receptor and AMPA-receptor currents were decreased in hippocampal CA1 region in APP[V7171] transgenic mice, which is associated with impaired NMDA-dependent LTP (Dewachter et al. Neurobiol Aging. 2007, Jul 26).

5. New potential players in L-LTP

a) As one of our aims is to identify proteins involved in L-LTP induction and in L-LTP, a prerequisite was to elaborate a technique capable to identify a protein modified as a result of synaptic activity.



With such an aim in mind, an L-LTP was induced chemically (in order to cause a change in a great number of synapses) by applying forskolin, an activator of adenylate cyclase and IBMX, an inhibitor of phosphodiesterase. The CA1 region was removed from each hippocampal slice using a razor blade under a dissecting microscope. Each CA1 sample intended to be submitted to further biochemical analysis was obtained by pooling the CA1 regions taken from six individual slices and provided 50 µg of protein. These proteins were then separated by two-dimension electrophoresis (2-DE). A modification of a protein occurring during L-LTP could then be spotted on these 2-DE maps. For instance, using anti-phosphotyrosine antibody, we detected a protein that was strongly phosphorylated on tyrosine during L-LTP. The spots corresponding to that protein could then be punched and further identified using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). In the case used as a trial, the phosphorylated protein spotted was found to be rabphilin, a protein present in presynaptic terminals. (Capron et al. Neurosci Lett 2007).

b) Synaptic plasticity can also be explored in cultured hippocampal neurons.The frequency of miniature excitatory postsynaptic currents (mEPSCs) in cultured hippocampal neurons increases under the action of drugs that trigger a rise in the intracellular concentration of cyclicAMP. It is generally believed that this type of effect is mediated by protein kinase A. We showed that it largely depends on the activation of hyperpolarization-activated cation channels (Ih) by cyclicAMP. In mammals, Ih channels control membrane excitability, thanks to their function as ionic channels. we show that the effect of Ih channels on glutamate release is not mediated by the depolarization induced by their activation and thus is not linked to the ionic channel aspect of Ih channels. This suggests that the Ih channel could be a bifunctional protein. (Genlain et al. Neurosci Lett 2007)

Publications with acknowledgments to the FMRE

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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 promoter 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) (Soriano 1999) 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, the reporter mice expressing Z/EG under the control of the pCAGGS promoter (Novak et al., 2000), demonstrated the expression of eGFP 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 (see figure 1) (de Kerchove d'Exaerde et al., 2006). In the same line of works, we have collaborated to the construction and generation of another line of mice expressing specifically Cre recombinase in the striatum (Lemberger et al., 2007).

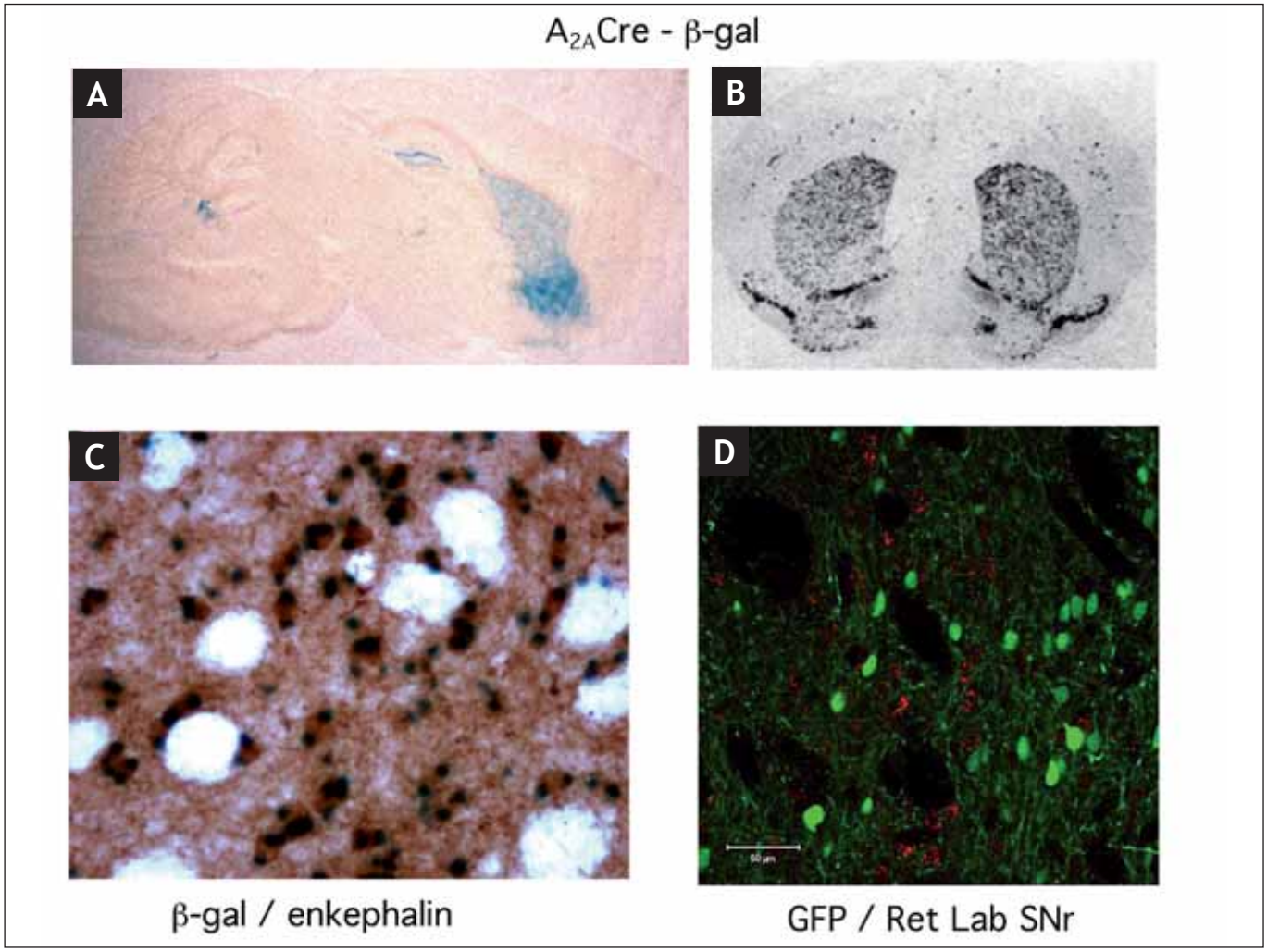

Figure 1: Reporter mice (Rosa26-lox-Stop-lox-LacZ (A,B,C) or pCAGGS-lox-STOP-lox-EGFP (D)) crossed with A_{2A}r-Cre mice were used to localize Cre-mediated recombination.
(A,B) β-galactosidase histochemistry (blue) shows a restricted staining in the striatum in parasaggital (A) and coronal sections (B). Co-labeling with enkephalin-immunocytochemistry (brown) (C) or excluded labeling of GFP (green) with the retrograde labeling of striatonigral neurons (red) (D) demonstrates the restricted recombination in striatopallidal neurons.

The striatopallidal-GFP mice has been used as a tool to specifically isolate this population of striatal neurons by using dissociation and sorting by FACS and to specifically record them by using the patch clamp technique. We succeed in isolating green (striatopallidal) and red (striatonigral) neurons from individual mice with a very good rate of enrichment. In this frame, our aim is now to establish the gene expression profiles of these different populations by microarrays. RNA amplification and identification will be performed to validate the differential expression of some known genes (i.e. enkephalin , substance P, A_{2A} receptor). Since in the same time, a competing team published similar results Lobo et al. (2006), we re-oriented our studies by examining the modifications of the specific gene expression profile in this striatopallidal population in pathological conditions.

We have crossed these "striatopallidal-Cre" mice 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 ($NR1^{f/f}$) mice has been previously generated and published (Tsien et al., 1996) 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 $A_{2A}R$ -Cre mice to obtain homozygous mice. The characterization of $A_{2A}R$-Cre/+ $NR1^{f/f}$ mice by binding autoradiography using a NMDA receptor specific ligand showed a 30% and 40% decrease in binding in the caudate-putamen and accumbens nucleus, respectively, as compared to wild type littermates. On the other hand, there was no differences in NMDA receptor ligand binding between mutant and wild-type in the cerebral cortex suggesting the selectivity of striatal NR1 inactivation.

In order to prevent any eventual compensation process that could result from gene inactivation started during development, we have initiated a second and alternative approach aiming to reversibly inactivate these striatopallidal neurons in a specific time window. For this, we used a second transgenic strategy, namely the TRE/tTA expression system which allow to inductively and reversibly control a gene expression in a specific cell population via administration of tetracycline (Kelz et al., 1999). This system is based on two components. On one hand, the gene coding for the transcriptionnal transactivator tTA (tetracycline TransActivator) is placed under the control of a cell-specific promoter. On the other hand, the gene whose expression will inactivate the neurons of interest is placed under the control of the TRE (Tetracycline-Responsive Element) promoter that binds tTA and will be regulated by the presence or absence of tetracycline. We have constructed and generated lines of transgenic mice that present a targeted expression of the transactivator tTA in striatopallidal neurons.

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 (D'Alcantara et al., 2001). We now studied 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. By using the perforated patch configuration of the patch clamp technique in combination with peptide occlusion protocols, we showed that dopamine D2 receptor activation abolished the NMDA-induced down- to up-state transitions and hence striatal neurons excitability. Peptide occlusion showed that this effect occurs through a pathway involving a subtype of calcium channels (CaV1.3). This D2-mediated effect is fully reversed by co-stimulation of A_{2A} receptor although activation of the A_{2A} receptor is unable to modify the down- to up-state transitions (Azdad et al., 2007). This suggests that the action of A_{2A} receptor activation could be completely or partially due to an intramembrane interaction such as D2-A_{2A} heteromerization rather than to activation of an intracellular cascade. Experiments have been performed to test this hypothesis by using occlusion by specific competitive peptides blocking A_{2A}-D2 heteromerization. We showed that such peptides fully blocked the ability of A_{2A} receptor activation to counteract the D2 effect demonstrating the involvement of D2- A_{2A} heteromerization in this modulation and supporting for the first time in a physiological condition, the physiological relevance of this heteromerization (Azdad et al., 2007).

Some of these results have been obtained in A_{2A} receptor knock-out animals (Azdad et al., 2007) and experiments are also conducted in transgenic rats overexpressing the human A_{2A} receptor that we fully characterized morphologically and behaviourally (Gimenez-Llort et al., 2007).

Neuronal death, neuroprotection and stem cell graft 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 (Blum et al., 2002, 2003a, 2003b). 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 (Bantubungi et al., 2005).

In the frame of these studies on therapeutics of neurodegenerative diseases, we have also addressed the question of cell therapy using stem cells. We have started the neurobiological characterisation of the migration and homing processes of neural stem cells (NSC) and mesenchymal stem cells (MSC) following their intracerebral grafts in vivo, in a rat model of Huntington's disease. We showed that both stem cells (NSC and MSC) exhibit a migration pattern significantly different in the degenerative condition as compared to intact brain (Bantubungi et al., 2007). Our results demonstrated that the host environment determine the final localisation of grated cells and hence their homing. We have identified a molecular mechanism involved in this homing process as the « Stem Cell Factor (SCF) – Kit receptor » system (Bantubungi et al., 2007). Indeed, we demonstrated that SCF expression is highly upregulated in the lesioned striatum as compared to the intact side and showed that NSC and MSC express the Kit receptor. Different technical approaches in vitro demonstrated that this SCF-Kit, ligand-receptor, system is functional in both cell types since recombinant SCF as well as protein extract from the lesioned striatum, increased phosphorylation of Kit and Erk, increased cell proliferation and cell migration (Bantubungi et al., 2007). Finally, we have identified the cells producing SCF as atypical cells expressing GFAP, nestin and doublecortin in the lesioned area and as precursors cells expressing doublecortin when they are in migration from the rostral migratory stream to the lesioned area (Bantubungi et al., 2007).

Identification of a new brain anti-opioid system

We took part to the identification of prolactin-releasing peptide and its receptor GPR10 as a new neuropeptidergic system exhibiting anti-opioid effects (Laurent et al., 2006).

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 (Gall et al., 2003). We extended these data by using different mathematical models and reviewed these results in a general perspective (Gall et al., 2005a). 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 (Roussel et al., 2006). 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 (Roussel et al., 2006). This suggests that subtle and local modifications in calcium buffering capacity could dramatically change the mode of neuronal coding.

To more deeply understand the place of these granule cells in the computational network of the cerebellar cortex, members of the laboratory take part to several studies. The first was reported last year and described the molecular mechanisms of long term potentiation induction at the mossy fiber-granule cell synapse (Gall et al., 2005b) A second study identified the molecular mechanisms of GABA modulation on granule cell potassium channels. In this work, thanks to the development of multiplex RT-PCR on single cell, we were able to identify inward rectifier potassium channels subtypes expressed in individual granule cells and to correlate these expression profiles with the electrophysiological data (Rossi et al., 2006). Our results suggest that, in granule cells, $GABA_B$ receptors can exert an inhibitory control over constitutive inward rectifier K^+-currents mediated by Kir2 channels.

The absence of calretinin in cerebellar granule cells constituted a main hypothesis consistent with the perturbations that we previously demonstrated in $Cr^{-/-}$ mice (Schiffmann et al., 1999; Gall et al., 2003; Cheron et al., 2005). To investigate this hypothesis, we specifically rescued by cell-specific transgenesis 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 (Bearzatto et al., 2006). 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 behaviour 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 (Bearzatto et al., 2006).

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, Servais et al., 2005a). We suggested that this oscillation was generated by Purkinje cells

whose behaviour 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 behaviours 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, Servais et al., 2005a). 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 (Servais et al., 2005a).

Since these alterations in neuronal excitability and network interactions have been detected in different transgenic animals, we also wanted to know whether similar alterations could be observed in animal models mimicking human pathological conditions. We have therefore developed different models of alcohol intoxication known to affect preferentially the cerebellum and whose a target could be the calcium homeostasis. We first showed adult mice chronically consuming ethanol present alterations of Purkinje cells discharge pattern and motor incoordination (Servais et al. 2005b). The second model aimed to understand the cerebellar alterations resulting from prenatal intoxication (fetal alcoholic syndrome, FAS). We demonstrated that the behavioural deficit detected at adulthood in these mice is not related to Purkinje cell death but rather to the dysfunction of surviving Purkinje cells (Servais et al., 2007). We showed that Purkinje cells have a decreased expression in protein kinase C gamma and a resulting diminution in voltage-dependent calcium currents. As a consequence, we also demonstrated the conversion of the long term depression at the parallel fiber- Purkinje Cell synapse into a long term potentiation. Exogenous activation of protein kinase C restores a normal level of voltage-dependent calcium currents in FAS Purkinje cells. These observations could explain the augmentation of Purkinje cells firing rate and the presence of the fast local field potential oscillation detected in vivo as well as the behavioural alterations (Servais et al., 2007). These results demonstrated that in FAS, learning deficits are not the consequence of the precocious death of a large number of Purkinje cells but is rather related to the presence of Purkinje cells dysfunction due to molecular alterations in surviving Purkinje cells.

In all these studies on cerebellar physiology, we noted a rather high difficulty to obtain reproducible results in terms of locomotor behaviour. We therefore analysed it carefully in mice from different ages and genetic backgrounds (Bearzatto et al., 2005). We detected significant differences that pointed out the need of carefulness in the choice of the adequate mouse models.

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Final Report of the Research Group of

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Evaluation of leptin antagonists for treatment of multiple sclerosis

1. Introduction

The crucial role of leptin in the long-term control of body weight is well established. This cytokine with hormone-like characteristics is mainly produced and secreted by adipocytes, and plasma protein levels positively correlate with body fat energy stores (1,2). Low leptin levels (like in leptin deficient ob/ob mice, or evoked by starvation) correlate with severe immune dysfunctions and an increased risk of infections (3,4). It has become clear that the hormone plays a role in both innate and adaptive immunity (reviewed in (5)). In innate immunity, leptin promotes secretion of inflammatory cytokines, and activation of macrophages, neutrophils, and natural killer cells. Functions in adaptive immunity include thymic homeostasis, naïve $CD4^+$ cell proliferation, and promotion of T helper 1 (T_H1) responses. Moreover, leptin can act as a negative signal for the expansion of $CD4^+CD25^{high}$ regulatory T cells (T_{Regs}), known to dampen immune reactions (6). Given its role in regulating $CD4^+$ T cell mediated immunity, leptin can contribute to the onset and progression of several T-cell controlled autoimmune diseases. Examples include Crohn's disease (7), rheumatoid arthritis (8), multiple sclerosis (9,10), and autoimmune hepatitis (11).

Leptin mediates its effects upon binding and activation of a single-membrane spanning leptin receptor (LR). The receptor, encoded by the db gene (12), is a member of the class I cytokine receptor family. Due to alternative splicing and ectodomain shedding, six LR isoforms have been identified (LRa-f): a long form (LRb or LRlo), four short forms (LRa,c,d,f), and a soluble form (LRe). All isoforms have an identical extracellular domain consisting of two so-called cytokine receptor homology (CRH) domains. CRH1 and CRH2 domains are separated by an immunoglobulin-like (Ig) domain, and followed by two membrane-proximal fibronectin type III (FNIII) domains. The CRH2 domain is necessary and sufficient for leptin binding (14). Despite the lack of any affinity for the ligand, the FNIII and the Ig domains are crucial for receptor activation (14,15). We combined data from our signalling-complementation experiments (15), a detailed mutagenesis study on leptin (16), mapping of ligand binding sites on the CRH2 (17) and Ig domains (18) and LR cysteine mutation analysis (19) to propose a hexameric leptin:LR model for the activated receptor complex (18). Based upon these insights, we developed a leptin mutein (S120A/T121A) with antagonistic properties.

2. Development of nanobodies targeting the leptin receptor

A possible concern in the development of leptin-based therapeutic strategies for autoimmune diseases is that complete LR blockage also interferes with leptin's central body weight regulating role. Indeed, treatment of mice with for example the S120A/T121A leptin mutant induces significant weight gain (16). It is likely that the blood-brain-barrier (BBB) can be exploited to separate the peripheral (i.e. on immune cells) and central (i.e. on hypothalamic neurons) activities of leptin. An active leptin BBB transport model has been suggested, but the transporter itself has not yet been identified (20). 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 aimed to develop a leptin antagonist that does not interfere with this 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 (see above), we postulated that molecules directed against the LR FNIII or Ig-like domains are good candidates to fulfil 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. These VHHs have several advantages over conventional antibodies: (i) the long complement determining regions allow deeper binding in protein-protein interfaces or the catalytic centre of enzymes, (ii) they are very stable towards extreme pH and temperature, (iii) they are relatively small, which allows better tissue penetration, and make them easier to manipulate genetically, (iv) they can be produced in bacteria, which allows cost-effectively production and purification.

We have generated a panel of VHHs by immunization of lamas with the extracellular part of the murine leptin receptor. mLR-specific nanobodies were enriched in a series of panning rounds and tested for their ability to block leptin binding and receptor activation. We found that part of the VHHs were able to block leptin-induced proliferation of LR-expressing Ba/F3 cells. These neutralising VHHs bind to the CRH2, Ig, or FNIII domains in the receptor. We found that only CRH2 binders can also block leptin binding to its receptor, thereby further supporting the concept that this domain is the binding determinant. The Ig and FNIII domains display no detectable affinity for the ligand, but the observation that VHH directed against these domains further supports their role in receptor activation. These data illustrate that it is possible to block leptin signalling without affecting receptor binding.

3. Effect of neutralising nanobodies on body weight resulting in new insights in leptin transport across the blood-brain-barrier

Based on previous experiments, we have selected three neutralising VHHs: 2.17 (against CRH2, interferes with leptin binding), 4.10 (against Ig), and 4.11 (against FNIII). These were converted into a bivalent format. Using a flexible Gly-Ser linker they were fused to the Alb1 VHH that targets mouse serum albumin. One advantage is that binding to endogenous serum albumin greatly prolongs half-life of the resulting VHHs in circulation in vivo. Recombinant proteins (2.17-Alb1, 4.10-Alb1, and 4.11-Alb1) retained their LR binding and neutralization properties, and were purified to homogeneity. We evaluated the effect of bivalent VHHs on body weight as follows: proteins were administered daily (40 µg in PBS) in 9 to 10 week old C57BL/6 mice for 14 days. Alb1 (also 40 µg per day) and PBS were used as negative controls. Mice were weighed on a daily basis.

Results in figure 1 illustrate that 4.10-Alb1 provokes a clear increase in body weight, and this in contrast to 2.17-Alb1 and 4.11-Alb1. This difference can be explained by the observations that (i) 4.10-Alb1 is more potent in neutralization *in vitro* (data not shown), and (ii) 4.10-Alb1 serum levels, as determined in a plate binding-assay, were significantly higher when compared to the other two (229 µg/ml vs. 54 and 85 µg/ml for respectively 2.17-Alb1 and 4.11-Alb1 – data not shown).

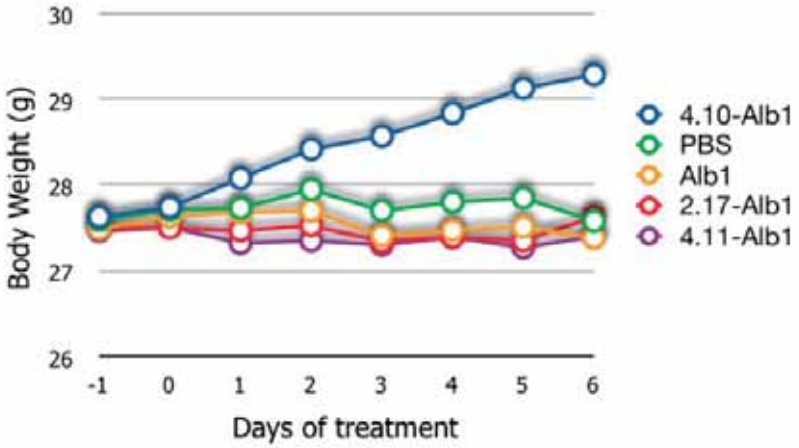

Figure 1: Effect of daily administration of bivalent VHHs on body weight

It is generally accepted that leptin needs to be transported across the BBB to exert its weight regulating effects. To date, the only two nanobodies know to cross this barrier were generated using human brain microvessel endothelial cells in the panning (21). Since it is highly unlikely that our bivalent VHHs are transported over the BBB, results with 4.10-Alb1 are puzzling. Possibly, they might support recent findings that (at least some) arcuate nucleus (ARC) leptin sensing neurons behave different from leptin-sensing neurons in the other sites of the hypothalamus (dorso-medial, ventro-medial, and lateral). First, diet-induced-obesity results in a decrease in leptin sensitivity due to over-expression of SOCS3 (a negative regulator of leptin signalling) in ARC neurons, but not in other regions of the brain (22). Second, it is only possible to detect basal STAT3 phosphorylation in the ARC neurons, and these respond more rapidly and sensitively to peripherally administrated leptin (23). Third, leptin-responsive neurons that express the LR or show STAT3 activation can be labelled by BBB impermeable fluorescent tracers (23,24). These observations can be explained by assuming a direct contact between the ARC neurons and the blood-circulation by for examples projections through the BBB. Data with our 4.10-Alb1 are in line with this hypothesis. Alternatively, we also cannot exclude at present that 4.10-Alb1 is actively co-transported by LR-mediated transcytosis into the brain. An implication from the former scenario is that it may be impossible to discriminate between leptin's peripheral and weight regulating central functions. Further studies, e.g. by *in vivo* tracking of peripherally injected 4.10-Alb1, are required.

4. Evaluation in a mouse model for multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease pathologically characterized by inflammation of the central nervous system (CNS) and the spinal cord. Demyelination and destruction of oligodendrocytes gives rise to severe paralysis and eventually death. In experimental autoimmune encephalomyelitis (EAE), a well characterized model for MS, disease can be provoked by immunization of susceptible mice with antigens of the CNS. Two models are currently being used: chronic-progressive (immunization with myelin oligendrocyte glycoprotein (MOG)) and relapsing-remitting (immunization with proteolipid protein peptide).

There is a growing body of evidence that leptin plays a crucial role in the onset and progression of MS: (i) leptin-deficient *ob*/*ob* mice are resistant to EAE, and leptin administration reverses this protection; (ii) a surge in leptin precedes the onset of EAE in both models; (iii) this increase in production is also observed in humans, and the use of leptin as a diagnostic marker was suggested; (iv) female mice are more susceptible to EAE than their male littermates; (v) *in vivo* leptin neutralisation improves the initiation, progression and clinical relapses in both EAE models.

We evaluated our antagonists in a chronic-progressive EAE model. C57BL/6J mice were injected subcutaneously with 200 µg MOG peptide at day 0. Starting at day 7 (i.e. just before the first clinical signs), mice received every other day an injection of (i) PBS; (ii) 4.10-Alb1, 100 µg/mouse; or (iii) pegylated leptin S120A/T121A mutant, 40 µg/mouse. Mice were scored daily according to the scale: 0.5 = partial tail paralysis; 1 = complete tail paralysis; 2 = unsteady gait; 2.5 = hind limb paralysis, animal is still able to move hind limbs; 3 = complete hind limb paralysis; 4 = paraplegia and/or quadraparesis; and 5 = moribund or death. Clinical scores are shown in figure 2.

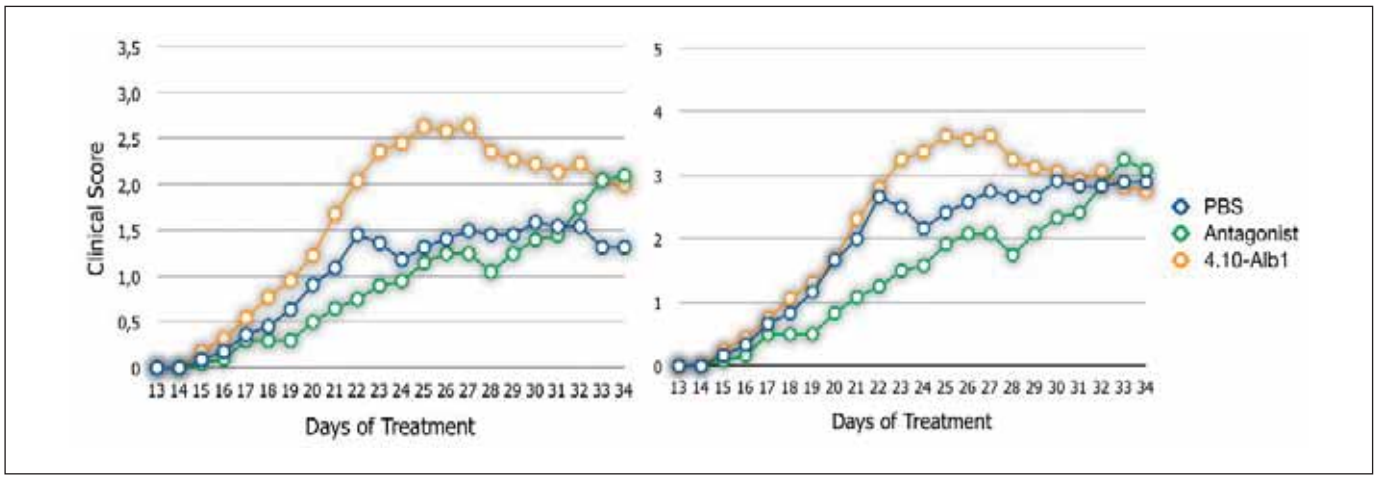

Figure 2: Clinical scores of PBS, nanobody, and antagonist treated mice. Left: all mice; right: diseased animals only

While a partial protection is observed in antagonist treated mice, administration of 4.10-Alb1 seems to worsen the clinical manifestations of MS.

5. Evaluation in ConA induced hepatitis

Hepatitis is commonly referred to as liver damage as a consequence of inflammation. It can be caused by viruses or toxins (like alcohol), or can be of auto-immune origin. Symptoms are typically flue-like, and the disease can lead to organ failure. A well-documented mouse model of T-cell dependent liver injury is the i.v. injection of the T cell mitogen concavalin A (ConA), which results in fulminant hepatitis (25). Leptin-deficient mice are protected from T-cell mediated hepatoxicity and display a decrease in TNF-α production (26). A more recent study illustrated that leptin and adiponectin, two important adipocyte-derived cytokines, have functionally opposite effects. While leptin increases sensitivity to ConA, adiponectin protects hepatocytes from cytokine-mediated cell death (27).

To evaluate the effects of 4.10-Alb1 on ConA induced hepatitis, C57BL/6J mice were treated with 100 µg/mouse/day of nanobody. On day 7, they received an i.v. injection of 200 µg ConA dissolved in pyrogen-free PBS. At different time-points after injection (0, 6, and 24 h), blood was collected from the retro-orbital plexus, and serum was prepared. Serum alanine aminotransferase (ALT) and aspartate aminotrasferase (AST) levels were measured and represent the severity of liver damage.

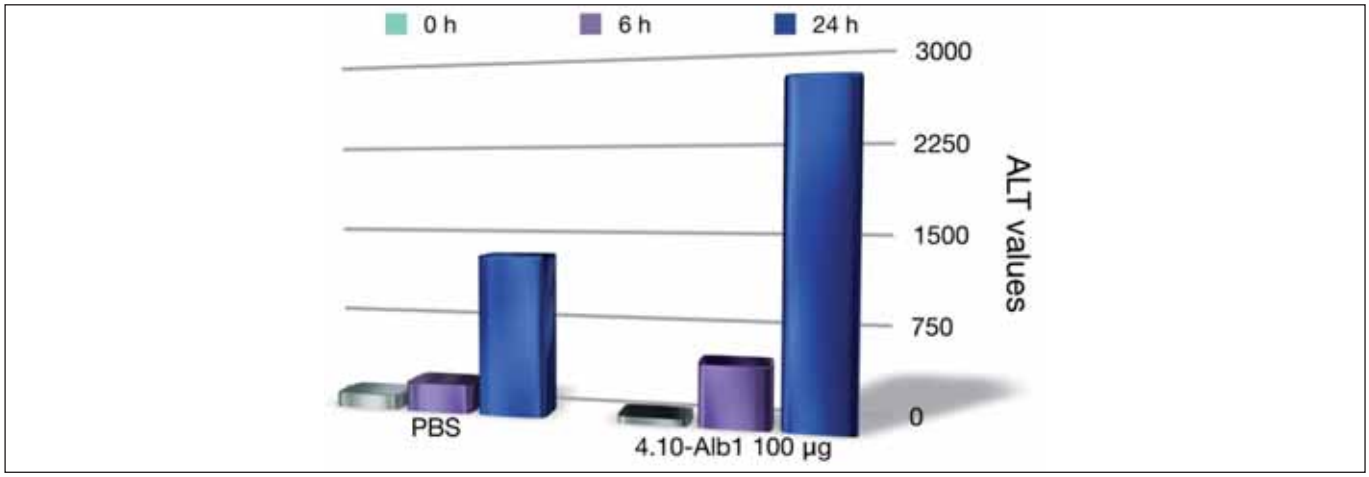

Figure 3: ALT measurements after ConA injection in PBS or 4.10-Alb1 treated mice

Rather unexpectedly but confirm with the EAE results, treatment with 4.10-Alb1 nanobody significantly increased liver damage. One possible explanation is that in the 4.10-Alb1 treated mice serum leptin levels are strongly elevated (17 vs 1,8 ng/ml, figure 4) (see higher). Leptin concentrations were measured with a mouse-leptin specific ELISA.

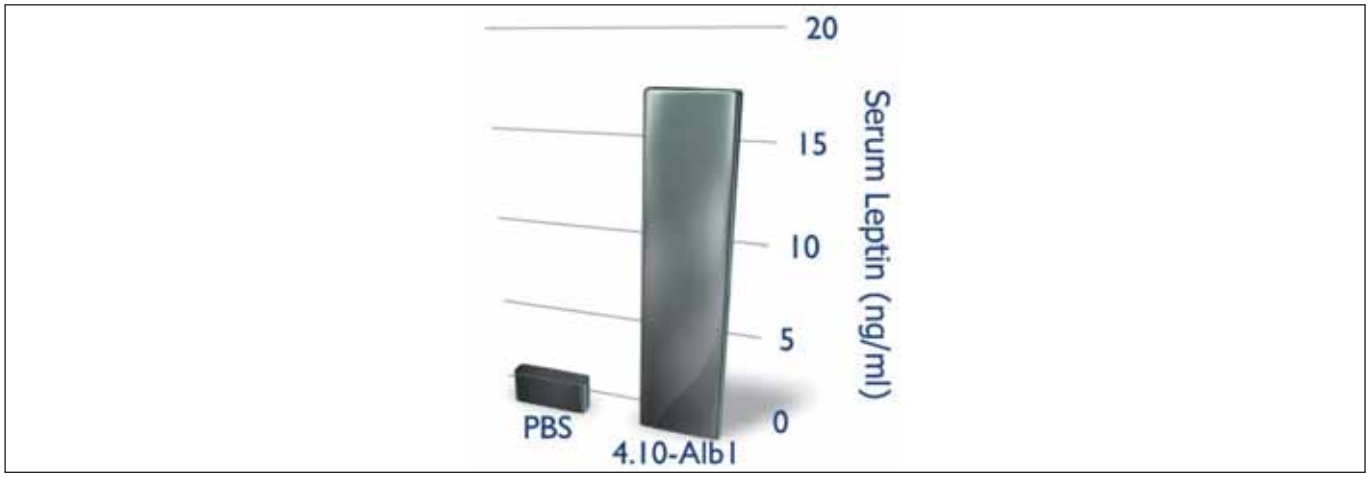

Figure 4: Serum leptin levels of PBS and 4.10-Alb1 treated mice

6. Conclusions

In conclusion, treatment with 4.10-Alb1 nanobody results in a significant worsening of the clinical outcome of ConA induced hepatitis and chronic-progressive EAE. For hepatitis, this can (at least in part) be explained by the elevated serum leptin concentrations. The question remains why 4.10-Alb1 does not suppress the immune-modulating functions of leptin. One explanation is that the levels 4.10-Alb1 are insufficient to neutralise the elevated leptin levels. Alternatively, leptin's immune effects might (at least in part) centrally mediated, which is likely inaccessible for nanobodies. This idea is supported by the observations with gold thioglucose (GTG) treated mice. GTG selectively destructs the hypothalamus, and has been used since long for treatment of hepatitis. GTG mice are obese (they weigh approximately twice as much as untreated mice), their serum leptin concentrations are elevated about ten-fold, bùt they do not develop significantly more liver damage in response to ConA. Given the selectivity of GTG, and the elevated serum levels, these data also suggest that leptin's effects on hepatoxicity are centrally mediated. Additional studies are planned to provide more insight into these unexpected effects of some leptin antagonists.

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Molecular genetics and biology of Charcot-Marie-Tooth neuropathies

We aimed to identify novel loci and genes for inherited peripheral neuropathies (IPN) through positional cloning and screening of positional and functional candidate genes. In addition, we performed mutation analyses in known and novel genes in families and isolated patients with a wide range of clinical Charcot-Marie-Tooth (CMT) phenotypes. We estimated the relative frequency of mutations, identified new mutations and revealed potential mutation hotspots. We performed detailed neurological investigations of patients with known and newly identified mutations. These analyses were important to determine if mutations are associated with a specific phenotype or involved in a broader disease spectrum. The identification of mutations was essential to plan functional studies to understand the effect of the mutation on protein function and interacting pathways. Here we summarize the novel genes we identified in the past 3 years of the GSKE project. Furthermore we will focus in this final report on the small heat shock protein genes (*HSP22* and *HSP27*) which we found to be causative for distal hereditary motor neuropathies (distal HMN).

Identification of 4 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 (NCV) 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, we and others have studied 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. 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. *DNM2* belongs to the family of large GTPases and is part of the cellular fusion-fission apparatus. This gene was identified in close collaboration with S. Züchner (Duke University, Durham, USA) (Züchner et al. 2005). For DI-CMTC on chromosome 1p35, we identified 2 missense mutations (G41R and E196K) and a *de novo* deletion of 4 amino acids in the tyrosyl-tRNA synthetase (YARS) in 3 unrelated families from Bulgaria, North America and Belgium. YARS is an essential enzyme for protein biosynthesis that catalyses aminoacylation of $tRNA^{TYR}$ with tyrosine. Biochemical experiments and genetic complementation in yeast demonstrated partial loss of aminoacylation activity of mutant proteins, and mutant YARS or its yeast orthologue TYS1 reduced yeast growth. YARS localizes in axonal termini of differentiating primary motor neuron and neuroblastoma cultures, and this distribution was not observed in cells expressing mutant proteins. YARS is the second aminoacyl-tRNA synthetase found to be involved in CMT (Jordanova et al. 2006).

We have previously shown that a missense mutation in *ARHGEF10*, coding a putative Rho GTPase guanine exchange factor (GEF), is associated with a dominant trait of slowed NCVs and thin myelin sheaths without an overt clinical phenotype in humans. To increase the understanding

of the role of Rho GTPase signalling in the peripheral nervous system, we combined positional and functional cloning strategies in collaboration with J. Senderek (RWTH, Aachen, Germany). We investigated critical intervals of CMT forms for which no disease genes had been identified so far for the presence of genes encoding Rho GTPases or Rho GTPase regulatory proteins. The most striking candidate was the *FGD4* gene that was identified within the critical interval for CMT4H on chromosome 12p11.21-q13.11. The *FGD4* gene encodes frabin, a GEF for the Rho GTPase Cdc42. CMT4H is an autosomal recessive demyelinating form of CMT with early onset and severe scoliosis. We identified homozygous mutations in the *FGD4* gene in two consanguineous families from Turkish and Lebanese origin. The mutations resulted in premature stop codon or a missense mutation in the homozygous state. Frabin has one F-actin binding domain, a Dbl homology (DH) domain in tandem with a pleckstrin homology (PH) domain, a FYVE finger domain and a second PH domain. The adjacent DH and PH domains are the typical signature of GEFs for Rho GTPases and therefore it is not surprising that frabin was found as a GEF for the Rho GTPase Cdc42 (Stendel et al. 2007). The FYVE and PH domains are known to bind to phosphoinositide phosphatases (PIPs). The presence of FYVE and PH domains in frabin also suggests a cooperation of frabin with another group of CMT proteins, i.e. the myotubularin related proteins MTMR2 and MTMR13. MTMRs are PIPs that control spatial and temporal phosphoinositide pools on cellular membranes. Interestingly, a functional relationship between RhoGTPase signalling and MTMR proteins is supported by the fact that myelin abnormalities (outfoldings of myelin in Schwann cells) are found in nerve biopsies of patients with *MTMR2* and *MTMR13* mutations. We observed similar irregularities of the myelin sheaths in the nerve biopsies of patients with *FGD4* mutations.

Hereditary neuralgic amyotrophy (HNA) is an autosomal dominant recurrent focal neuropathy. The clinical hallmark of HNA is recurrent attacks of brachial plexus neuritis, with severe pain in the affected shoulder and arm followed by peripheral nerve palsies and muscle atrophy. These are often precipitated by infections, immunizations, parturition or strenuous use of the affected limb. Recovery occurs over weeks to month and is sometimes incomplete. The disease is often associated with mild dysmorphic facial features such as hypotelorism (close set eyes) and epicanthal folds ("Mongolian folds"). We previously delineated the critical HNA region on chromosome 17q25 by genetic fine mapping of informative recombinants and allele sharing resulting in a refinement of the locus to a ~600kb candidate region. This region contained only two 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. 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 (Kuhlenbäumer et al. 2005). Septin 9 is expressed in the motor and sensory part of the peripheral nervous system and at a particularly high level in the immune system. During mouse embryogenesis high expression levels of Septin 9 have been found in structures forming the face. This finding might explain the presence of dysmorphic features. Further studies are required to

obtain final prove of the causative nature of the mutations and to elucidate the pathomechanism underlying HNA. We hope that the elucidation of the pathomechanism underlying HNA, a potentially immune mediated relapsing inherited neuropathy, will also shed light on the pathomechanisms of other, more common, non-hereditary, recurrent peripheral neuropathies.

Study of mutations in small heat shock protein genes (HSP22 and HSP27) in distal HMN:
Distal hereditary motor neuropathy (distal HMN) is characterized by the predominant loss of motor neurons and their long axons in the peripheral nervous system. Usually in childhood or adolescence, distal HMN patients present a classical distal muscular atrophy syndrome characterized by progressive muscle weakness and atrophy of muscles of lower legs and feet. Distal HMN comprises a clinical and genetic heterogeneous group of diseases that are subdivided in several subtypes according to the mode of inheritance, age at onset, and the distribution of muscle weakness. Apart from the absence of sensory abnormalities, distal HMN closely resembles axonal Charcot-Marie-Tooth disease (CMT2). Selective or predominant degeneration of neurons of the motor system is also a pathologic feature of sporadic amyotrophic lateral sclerosis (ALS), the well-known and devastating motor neuron disease. In contrast to distal HMN, sporadic ALS is considered to be a complex disorder caused by diverse genomic and environmental risk factors. The underlying mechanisms of the preferential degeneration of motor neurons in distal HMN and ALS remain unclear. One strategy to reveal the nature of these disease processes is the identification of mutant genes in hereditary forms of disease.

To date, mutations in eight different genes have already been associated with distal HMN (Irobi et al. 2006). To study the mutation distribution of these genes, we performed a mutation analysis of six genes for autosomal dominant distal HMN in a cohort of familial and isolated patients with a predominant motor neuropathy (n = 112). Nine disease-causing mutations were identified in *HSP22*, *HSP27,* the *Bernardinelli-Seip congenital lipodystrophy 2* gene (*BSCL2*) and in the *senataxin* gene (*SETX*) in 17 patients. The study confirmed that two hot-spot mutations in *BSCL2* are the most common causes of distal HMN and related motor neuropathies. In addition, we provided evidence for a genetic mosaicism in transmitting the C-terminal *HSP27* mutation. This study validates and extends the genetic and phenotypic heterogeneity of distal HMN (Dierick et al. in press).

Human genetics and experimental models strongly support a critical role for *HSP27* in motor neuron biology and disease. Therefore, we analyzed the genetic contribution of *HSP27* to the etiology of ALS. Three rare genetic variations, one of which was located in the heat shock element (HSE) in the *HSP27* promoter, were identified in a cohort of 150 sporadic Belgian ALS patients. We demonstrated that the HSE-variant, found in one ALS patient, impaired the stress response of *HSP27* in neuronal cells by reducing heat shock transcription factor binding. This study demonstrates a complex role for HSP27 in motor neuron disease, ranging from a primary cause in distal HMN to a possible rare susceptibility factor in ALS (Dierick et al. 2006).

Articles in International Journals with acknowledgement to GSKE:

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Research Activities 2005-2006-2007:

Scientific Prizes:

- **De Jonghe, P.:** Three-yearly award (2006) Prof. Dr. Raymond Van den Bergh for Neurology, Neurosurgery en Psychiatry. Vereniging van Vlaamse Zenuwartsen (VVZ). "Clinical, electrophysiological and molecular genetic aspects of inherited peripheral neuropathies".
- **Timmerman, V.:** Prijs van de Stiching Antoine Faes voor onderzoek in Biomedische Wetenschappen, Turnhout, 5 oktober 2006: "Inherited peripheral neuropathies: identification of loci and genes through molecular genetic and biological research".
- **Timmerman, V. en De Jonghe, P.:** GSKE - Solvay Prize (2007): "Moleculaire genetica en biologie van Charcot-Marie-Tooth neuropathieën", Brussel, 22 mei 2007
- **Irobi, J.:** Laureaat van de Tweejaarlijkse Prijzen van de Onderzoeksraad Universiteit Antwerpen, De Prijs Vandendriessche, 7 december 2005
- **Irobi, J.:** Koninklijke Academie voor Wetenschappen - Frans Van Cauwelaertprijs 2006-2007: 'Moleculair genetische en functionele analyse van mutaties in genen geassocieerd met erfelijke motorische neuropathieën', Brussel, November 21, 2007
- **Irobi, J.:** Koninklijke Academie voor Wetenschappen - Prijs van de Vlaamse Wetenschappelijke Stichting voor Biomedische Wetenschappen 2007: 'Moleculair genetische en functionele analyse van mutaties in genen geassocieerd met erfelijke perifere neuropathieën', Brussel, December 1, 2007

Awards and fellowships:

- **Claeys, K.:** Fellowship of the World Muscle Society, Brugge, October 4-7, 2006
- **Dierick, I., Irobi, J. and Verhoeven, K.:** Travel Awards of the Fund for Scientific Research (FWO-Vlaanderen) to attend the Conference on Molecular and Cellular Mechanisms of Axon Degeneration, The Babraham Institute, Cambridge, UK September 11-12, 2006
- **Dierick, I. And Baets, J.**: Charcot Marie Tooth Association (CMTA) fellowships to attend the Second International CMT Consortium Meeting, Snowbird, Utah, USA, July 18-20, 2007
- **Janssens, S.**: Marie Curie Conferences and Training Courses, supported by the European Commission, fellowship award to attend the 3rd Neurotrain Training Course, Dubrovnik, Croatia, October, 19-24, 2007
- **Verhoeven, K.**: FWO travel award to attend the EMBO/FEBS Workshop on Systems Dynamics of Intracellular Communication - Overcoming Distance in Signalling Networks, Ma'ale Hachamisha, Judean Hills, Israel, March 18-22, 2007

PhD theses:

- **Nathalie Verpoorten, MSc:** Identification of novel genes for primary sensory neurons and Molecular genetics of sensory and motor neuropathies. PhD in Biomedical Sciences. Public defence: 25/01/2006
- **Gregor Kuhlenbäumer, MD:** Identification of the genetic defect causing hereditary neuralgic amyotrophy (HNA). PhD in Biomedical Sciences. Public defence: 27/10/2006
- **Ines Dierick, MSc:** Identification and characterization of mutations in HSP27 and other genes associated with motor neuropathies. PhD in Biomedical Sciences. Public defence: 17/12/2007

Master theses:

- **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.
- **Ellen Claesen:** Mutatie- en koppelingsanalyse in families met erfelijke motorische neuropathieën. Academic Year 2005-2006. Master in Biology.
- **Stefaan Felix:** Mutatieanalyse van het DCTN1-gen in patiënten met een distale hereditaire motorische neuropathie. Academic Year 2005-2006. Graduation report.

Invited Lectures at international meetings:

- De Jonghe, P.: 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
- De Jonghe, P.: "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
- De Jonghe, P.: "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

- **De Jonghe, P.**: "Hereditary motor neuropathies ", 11th International Congress on Neuromuscular Diseases. Istanbul, Turkey, 2-7 July, 2006
- **De Jonghe, P.**: "New horizons in hereditary neuropathies", 11th International Congress on Neuromuscular Diseases. Teaching course. Istanbul, Turkey, 2-7 July, 2006
- **De Jonghe, P.**: "The never ending story of genetics of inherited neuropathies". 11th International Congress of the World Muscle Society: Brugge, October 4-7, 2006
- **De Jonghe, P.**: 5th EPNS Teaching Course – Neuromuscular Disorders: SMA and neuropathies, Kusadasi, Turkey September 23-25 (2007)
- **De Jonghe, P.**: 7th European Congress of the European Pediatric Neurology Society and the 9th Congress of the Turkish Child Neurology Society: Plenary Session - Hereditary Neuropathies, Kusadasi, Turkey September 26-29 (2007)
- **Timmerman, V.**: "Progrès Récent dans la génétique moléculaire des maladies de Charcot-Marie-Tooth", Congrès CMT-France, Lille, France, March 19, 2005
- **Timmerman, V.**: "Molecular genetics and biology of inherited peripheral neuropathies". Kongress: Diagnose und Management von Gangstörungen als interdisziplinäre Herausforderung, Graz, Austria, September 30 - October 2, 2005
- **Timmerman, V.**: "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
- **Timmerman, V.**: "About nerves, pedigrees and genes: molecular genetics of inherited peripheral neuropathies", Symposium on Neuromuscular Disorders: VI Simposio internazionale di genetica clinica e molecolare – Basi genetico-molecolari di malattie neuromuscolari:, Rome, Italy, May 19, 2006
- **Timmerman, V.**: "Aminoacyl-tRNA-synthetases in health and disease", "About nerves, pedigrees and genes: molecular genetics of inherited peripheral neuropathies" Research Seminar, The Scripps Research Institute, San Diego, CA USA, June 2-7, 2006
- **Timmerman, V.:** "About nerves, pedigrees and genes: novel insights in the molecular genetics of Charcot-Marie-Tooth neuropathies". Seminar at La Salpêtrière Hospital:, Paris, France, January 26, 2007
- **Timmerman, V.:** "Hereditary Neuropathies: Distal hereditary motor neuropathies (dHMN) / distal spinal muscular atrophies (dSMA)", International Symposium on Neurogenetics: Freiburg, Germany, February 28 – March 1, 2007
- Timmerman, V.: "Molecular genetics of hereditary neuropathies: an update", Joint Meeting Belgian-Dutch Neuromuscular Study Club and German Reference Center for Neuromuscular Diseases of the DGNN, Vaals, The Netherlands, March 23-24, 2007
- **Irobi, J.**: "About nerves, pedigree and genes: Molecular Genetics of Inherited Peripheral Neuropathies". Faculty of Medicine Seminar, University of Ibadan, Nigeria, August 16, 2006
- **Irobi, J.**: "Molecular genetics and biology of small heat shock proteins causing inherited peripheral neuropathy", 2nd World Conference of Stress: Budapest, Hungary, August 23-26, 2007
- **Jordanova, A.**: "Aminoacyl-tRNA-synthetases in health and disease, Disrupted function and axonal distribution of mutant tyrosyl-tRNA synthetase in dominant intermediate Charcot-Marie-Tooth neuropathy". Research seminar at The Scripps Research Institute, San Diego, CA USA, June 2-7, 2006
- **Jordanova, A.**: "Involvement of AARSs in disease and therapeutics, Disrupted function and axonal distribution of mutant tyrosyl-tRNA synthetase in dominant intermediate Charcot-Marie-Tooth

neuropathy". International Conference on Aminoacyl-tRNA-synthetases: From the Genetic Code to Human Disease & Medicine, San Diego, CA USA, October 1-6, 2006

Invited Lectures at national meetings:

- **Timmerman, V.**: "Molecular genetics and biology of inherited peripheral neuropathies: a "nerve" ending story?" Seminar at the Centrum Medische Genetica, Universiteit Gent, 28 November 2006
- **Timmerman, V.**: "A general overview on the molecular genetics and biology of peripheral neuropathies." Research seminar at the Friedrich-Schiller-University Jena, Department of Clinical Chemistry: Jena, Germany, 13 September 2007
- Timmerman, V.: "Overview on hereditary motor neuropathies and hereditary sensory and autonomic neuropathies." Annual meeting of the the Spanish Society of Neurology - Neuromuscular Group session: Barcelona, Spain, 23 November 2007
- **De Jonghe, P.:** "Erfelijkheid bij ALS", ALS-Liga België, Contactweek-end, Oostende, 7-9 September, 2007
- **Irobi, J.:** "Functional characterization of HSP22 mutations associated with axonal degeneration", VIB Science Club Neurodegenerative Diseases, Leuven, 6 October 2006
- Irobi, J: "Moleculaire genetica en functioneel onderzoek van mutaties in HSPB8 geassocieerd met erfelijke motorische zenuwaandoeningen." Lezing naar aanleiding van de prijsuitreiking van de prijs van het Frans Van Cauwelaert fonds 2007; Koninklijke Vlaamse Academie van België voor Wetenschappen en Kunsten, Brussel, 21 November 2007
- **Rotthier, A.**: "Muizenissen in CMT", CMT-Dag, UA, Antwerpen, 31 March 2007
- **Baets, J.:** "Zin en onzin van het EMG in CMT", CMT-Dag, UA, Antwerpen, 31 March 2007

Slide presentations selected at international meetings:

- **Timmerman, V.**: "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
- Timmerman, V.: "Mutations in the FGD4 gene encoding the Rho GTPase guanine nucleotide exchange factor frabin cause autosomal recessive Charcot-Marie-Tooth neuropathy type 4H" The 2nd International CMT Consortium, Snowbird, Utah, July 18-20, 2007.
- **Claeys, K.**: "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
- **Claeys, K.**: "Mutations in mitofusin 2 are a major cause for autosomal dominant axonal Charcot-Marie-Tooth neuropathy". International Symposium on Neuromuscular diseases, Brussels, October 14-15, 2005
- **Claeys, K.**: "Dynamin 2 mutations are associated with dominant intermediate Charcot-Marie-Tooth disease and dominant centronuclear myopathy", 11th International Congress of the World Muscle Society:, Brugge, October 4-7, 2006
- **Irobi, J.**: "*In vitro* expression of small heat shock protein HSPB8 and HSPB1 mutations causing axonal neuropathy". 6th Dutch Chaperone meeting, UMCG Groningen, The Netherlands, February 24, 2006
- **Irobi, J.**: "*In vitro* expression of small heat shock protein HSPB8 and HSPB1 mutations causing axonal neuropathy", 11th International Congress of the World Muscle Society, Brugge, October 4-7, 2006
- **Irobi, J.**: "*In vitro* expression of small heat shock protein HSPB8 and HSPB1 mutations causing axonal neuropathy". 6th Bi-annual Meeting of the Belgian Society for Neuroscience, Brussels, May 20, 2005

- **Irobi, J.**: "*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
- **Irobi, J.**: "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
- **Nelis, E.**: "Molecular genetic diagnostics of polyneuropathies. Neuromuscular Meeting – Polyneuropathies", Helsinki, Finland, November 17, 2006
- **Jordanova, A.**: "Disrupted function and axonal distribution of mutant tyrosyl-tRNA synthetase in dominant intermediate Charcot-Marie-Tooth neuropathy". European Conference of Human Genetics 2006, Amsterdam, The Netherlands, May 6-9, 2006
- **Jordanova, A.**: "DI-CMTC associated mutations give rise to proteins with different enzymatic activity and physical characteristics". The 2nd International CMT Consortium, Snowbird, Utah, July 18-20, 2007
- **Janssens, S.:** "The role of the innate immune system in peripheral neuropathies", Seminar: Les Crosets, Switzerland, 23 January 2007
- **Dierick, I.**: "Small heat shock proteins in motor and sensory neuron disease". 2nd International Congress on Stress Responses in Biology, Tomar, Portugal, September 24-28, 2005
- **Dierick, I.**: "An ALS-related variant in the SSPB1 promotor impairs the HSP27 stress response". The 2nd International CMT Consortium, Snowbird, Utah, July 18-20, 2007
- **Dierick, I.**: "Mutation distribution of known genes for autosomal dominant distal hereditary motor neuropathies: a genotype – phenotype correlation study", Belgisch Nederlandse Neuromusculaire Studieclub, Utrecht, September 26, 2007
- **Verpoorten, N.:** "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
- **Baets, J.**: "Peripheral neuropathy and 46XY gonadal sysgenesis: a heterogenous clinical entity". The 2nd International CMT Consortium, Snowbird, Utah, July 18-20, 2007

Slide presentations selected at national meetings:

- Irobi, J.: "Functional studies of small heat shock proteins causing inherited axonal neuropathy". Belgian Society for Neuroscience, University of Antwerp, May 7, 2007
- **Jordanova, A.**: "Disrupted function and axonal distribution of mutant tyrosyl-tRNA synthetase in dominant intermediate Charcot-Marie-Tooth neuropathy". VIB Seminar 2006, Blankenberge, March 9-10, 2006
- **Dierick, I.**: "An ALS-related variant in the HSPB1 promotor impairs the HSPB1 heat shock response". VIB Seminar 2007, Blankenberge, March 1-2, 2007

Patents:

- **European Patent Application EP 05109485.2:** Diagnostic test for the detection of dominant intermediate Charcot-Marie-Tooth neuropathy. Applicant: Flanders Interuniversity Institute for Biotechnology (VIB), Inventors: Jordanova A., Thomas F., De Jonghe P. & Timmerman V., Date of Filing: 12-10-05

Final Report of the Research Group of

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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 and from mouse to man, 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).

We have summarized below the work achieved over the past years thanks to the Funding of the FMRE/GSKE, providing a link with our previously published work, as well as its perspectives in the future.

1. Multiple roles for ephrin/Eph genes in the development of the cerebral cortex.

We previously demonstrated that ephrin-A5 and its receptor EphA4 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). Taking advantage of this system, we have now investigated in vivo the signalling mechanisms of neural mapping mediated by the EphA4 receptor, previously shown to control topographic specificity of thalamocortical axons in the mouse somatosensory system. Using axon tracing analyses of six knock-in mouse lines displaying selective mutations for the *ephA4* gene (obtained in collaboration with R. Klein, MPI Munich), we determined for the first time which intracellular domains of an Eph receptor are required for topographic mapping (Dufour et al., 2006;Egea et al., 2005). We obtained direct in vivo evidence that the tyrosine kinase domain of EphA4, as well as a tight regulation of its activity, are required for topographic mapping of thalamocortical axons, while non catalytic functional modules such as the PDZ binding motif (PBM) and Sterile-α motif (SAM) domain are dispensable. These data provide novel insight into the molecular mechanisms of topographic mapping, and constitute a physiological framework for the dissection of the downstream signalling cascades involved (Dufour et al., 2006).

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 EphA7 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). These 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, as was recently confirmed by other groups (Noren et al., 2006). We have followed 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. On the one hand we have extended our observations by studying compound knock-out lines for ephrin-A2, ephrin-A3 and ephrin-A5 compound genes. Importantly we found in these mutants an even more robust appearance of exencephalic forebrain overgrowth previously observed in EphA7 knock-out mice (Depaepe et al., 2005), together with a reduction of apoptotic rates, not only in the cortex, but also in the ventral part of the forebrain, thus enabling us to conclude that ephrin-mediated apoptosis is a more general phenomenon during brain development (V. Depaepe and P.V., unpublished data). In addition we have obtained evidence that ephrin-dependent apoptosis is essentially observed on proliferating neural progenitors, while caspase 3 activation, but not cell death, is observed following ephrin stimulation of post-mitotic neurons. These data provide first insights on the cellular mechanisms of ephrin-dependent apoptosis, that seems to be highly dependent of the cellular context, which will be studied further using cortical progenitors and neurons in vitro.

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.

In vivo and in vitro approaches are being used to address this important question. 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. In parallel we have developed an in vitro organotypic model combined with electroporation, in order to study the regulatory sequences responsible for the control of transcription of ephrin-A5 and EphA7. This system has recently enabled us to identify a 6 kb fragment of the EphA7 promoter, that seems necessary and sufficient to direct expression of a GFP reporter to cortical progenitors, and not to cortical neurons or cells of more ventral or caudal domains of the developing brain, which corresponds to the physiological pattern of expression of EphA7 at early stages of development (Depaepe et al., 2005). These findings are now being followed up by transgenesis in vivo, by generating transgenic mice with the same constructs.

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 striatum. 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. In addition, the mammalian striatum can be subdivided into two morphologically and functionally defined compartments, the matrix and the striosomes, which underlies important functional features of the striatum. Matrix and striosome neuronal populations are generated sequentially from the LGE during embryonic development and segregate from each other to form distinct compartments at early perinatal stages, but the mechanisms involved in the control of migration and sorting of striatal neurons remain largely unknown.

We designed a novel organotypic assay to study striatal compartmentation, where eGFP+ neurons, taken at different embryonic ages, are dissociated on top of early postnatal slices to test for their capacity to recognize the presumptive matrix and striosom compartments,. This assay allowed us to identify ephrin/Eph family members as candidate factors regulating matrix/ striosome compartmentalization. This was further confirmed in vivo by the analysis of the striatal patterning in ephrin-A5/EphA4 compound mutant mice, which revealed that ephrin-A5/EphA4 genes are required in vivo for the proper sorting of matrix and striosome neuronal populations. These data constitute the first identification of guidance genes involved in the control of striatal patterning, supporting a model whereby the temporal control of neuronal guidance cues enables the development of the specific cytoarchitecture of this structure (Passante et al., 2008).

In parrallel we have set up organotypic assays to dissect the molecular and cellular mechanisms that control the migration of distinct populations of neurons to dorsal vs ventral 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-B1-2 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 particular ephrin-B1-2 conditional kos, available in the laboratory), 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 from embryonic stem cells.

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 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 developed an in vitro model of neural differentiation of embryonic stem (ES) cells to study the specification of cortical neurons (Gaspard et al., 2007). Using a chemically defined medium devoid of any exogenous morphogen factors, we found that mouse ES cells cultured as monolayers spontaneously and efficiently (>80%) give rise to a population of neural precursors expressing regional markers indicative of a forebrain identity. When exposed to appropriate morphogen antagonists during their differentiation, in particular inhibitors of the *Sonic-Hedgehog* pathway, the fate of the ES cell-derived forebrain-like neural progenitors can be efficiently (>75%) directed to an identity corresponding to the cortical lineage, expressing Pax6, Tbr2, Emx1-2, Otx1-2 and Fox-G1 transcription factors.

ES cell-derived cortical-like progenitors subsequently differentiate into a stereotyped population of neurons, most of which (>75%) display all landmarks of cortical pyramidal neurons, including a glutamatergic phenotype and a pyramidal morphology. Most strikingly, ES cell-derived cortical neurons sequentially express a defined repertoire of markers that correspond to distinct subtypes of cortical neurons, in a manner strikingly similar to the in vivo situation: cells expressing markers of Cajal-Retzius neurons are first being generated, follwowed by pyramidal neurons expressing markers of deep cortical layers, and eventually by neurons expressing markers of the superfical layers. Finally ES cell-derived cortical neurons can effectively integrate into postnatal cortical tissue, display electrophysiological and synaptic activity profiles indistinguishable from excitatory cortical neurons, and, most importantly, can connect with the rest of the brain in vivo like genuine cortical projection neurons..

Our results indicate that ES cells can be efficiently specified into cortical neural progenitors and pyramidal neurons in a chemically defined medium lacking any morphogen and containing only morphogen inhibitors, thereby surprisingly suggesting a "default pathway" of cortical neuron specification. This model of in vitro "corticopoiesis" recapitulates all milestones of cortical development observed in vivo and therefore constitutes an attractive and robust system to be used for the genetic dissection of the mechanisms of cortical neuron specification. It could be also used as a robust source of cortical neurons for pharmaceutical screens or pave the way for the rational design of cellular therapies of the cerebral cortex.

We have 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 will be also invaluable for our further studies on human cortical development.

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 developing human brain, trying to reveal what are the specific developmental programmes underlying the emergence of human-specific features in our brain.

This project first 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).

More recently we identified, in collaboration with the bioinformatic group of D. Haussler (HHMI UCSC) that HAR1 (*Human accelerated Region 1*), a novel non coding RNA gene that is highly expressed in the developing cortex and has undergone a dramatic evolutionary acceleration in the human lineage (Pollard et al., 2006). We were able to show that HAR1 is strongly expressed in the human embryonic neocortex, in particular in Cajal-Retzius (C-R) neurons, a transient cell population, particularly prominent in primates, that plays a major role in the control of cortical paterning from mice to man (Amadio and Walsh, 2006). In addition HAR1 displays a novel and highly conserved secondary structure of unknown function (Pollard et al., 2006), that makes it an attractive candidate for regulation of gene expression, similarly to miRNAs.

Given its potential involvement in the development and evolution of the cerebral cortex, we are now focusing on the function of HAR1. On one hand we are undertaking a gain-of-function approach, using electroporation of human and mouse HAR1 expression constructs in mouse embryonic brain (by *ex vivo* and *in utero* electroporation, both used routinely in the lab), looking for alterations in neuronal migration, differentiation, and connectivity. Particular attention will be focused on potential differences between effects of mouse and human HAR1. On the other hand, we are developing in vivo transgenic approaches to generate HAR1 gain and loss of function. We have started to generate knock-out mice for the HAR1 gene using classical homologous recombination in ES cells, targeting the ultraconserved domain of HAR1 present in the mouse, and replacing it by a Cre- excisable selection cassette (targeted Es cells have been generated, chimeras are being produced). In parallel we are generating a gain of function line by knocking a condtional (Cre dependent) HAR1 expression construct into the ROSA26 locus (Belteki et al., 2005). Mice derived from recombined HAR1/ROSA26 ES cells will allow robust and conditional expression of HAR1 in vivo following appropriate crosses with Cre expressing mice such as Emx1, FoxG1 and Dbx1 Cre mice. Both gain- and loss-of-function conditions will be analyzed, looking for potential changes in neural proliferation, migration, specification, and connectivity using techniques routinely used in the lab (Depaepe et al., 2005;Dufour et al., 2003). Together with the study of HAR1 in neurons derived from human ES cells, this work may enable us to uncover the biological function of this gene that may have played a crucial role in human brain evolution.

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- Ephrin signalling controls brain size by regulating apoptosis of neural progenitors.
  Depaepe V, Suarez N, Passante L, Dufour A, Gorski J, Jones K, Ledent C, and Vanderhaeghen P.
  Nature 435 (2005), 1244-1250.*(IF 30)*
- Mapping labels in the developing human visual system and the evolution of binocular vision.
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- Regulation of EphA4 Kinase Activity Is Required for a Subset of Axon Guidance Decisions Suggesting a Key Role for Receptor Clustering in Eph Function.
  Egea J, Vig Nissen U, Dufour A, Sahin M, Greer P, Kullander K, Mrsic-Flogel T, Greenberg ME, Kiehn O, Vanderhaeghen P, Klein R.
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  Nature 443 (2006),167-172. *(IF 30)*
- Genetic Analysis of EphA-Dependent Signalling Mechanisms Controlling Topographic Mapping in Vivo.
  Dufour A, Egea J, Kullander K, Klein R, and Vanderhaeghen P.
  Development 133 (2006), 4415-4420. *(IF 8)*

Final Report of the Research Group of

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Interactions between areas investigated using awake monkey (f)MRI.

During the past GSKE funding cycle we mainly aimed to investigate functional interactions between brain regions using fMRI in awake monkeys. We simultaneously combined fMRI with other invasive techniques such as intracortical electrical microstimulation and reversible deactivation by cooling. We were able to develop these challenging combinations of techniques and started to use them in several passive viewing and task experiments. Below, I describe the results of two such experiments (one pure task fMRI experiment; one combined microstimulation-fMRI experiment) which haven't been discussed in past reports of the GSKE. Importantly, a new 3T scanner, for experimental animal research became on-line during the last year of this funding cycle. As usual, it took a little longer than anticipated to turn the 3T into a fully operational apparatus. With the addition and help of Dr. H. Kolster who is expert in coil building and image processing, and several new graduate students in our laboratory, we are about to resume at full speed our monkey fMRI experiments at high magnetic field. Several manuscripts for which GSKE funding was used are currently (re) submitted (4) for publication and several others are being prepared for submission.

1. Searching for a salient target involves mainly frontal regions (Claire Wardak; Guy Orban)

Searching for a particular object in a visual scene involves selection mechanisms. Traditionally it is assumed that efficient search (pop-out) involves mainly bottom-up processing, whereas inefficient search requires attentional top-down control over visual processing. Functional magnetic resonance imaging (fMRI) in behaving monkeys is particularly suited to identify and to assess the relative contribution of visual, frontal and parietal areas involved in search. Monkeys were trained to covertly detect the presence of a salient target amongst distractor objects. Three control tasks, matched for visual input and motor responses, were used to control for the spatial allocation of attention. Contrary to mainstream thinking, efficient search activated three frontal regions, the frontal eye field (FEF), area 45 and a posterior portion of area 46, in addition to small activation sites in LIP and TE. No other visual areas were activated. Area 45 is specifically activated during a switch between tasks, i.e. when subjects have to reset what to respond to. Our data suggest that significant top-down control is exerted during efficient visual search and point to the central role of area 45 in this top-down control.

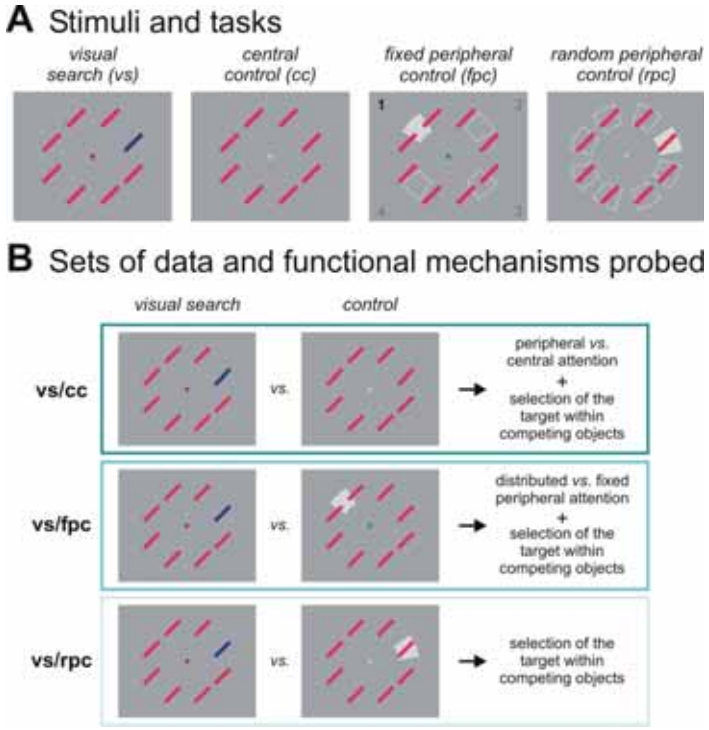

Figure1: Stimuli, tasks and functional mechanisms probed. A, Examples of stimuli for the 8-item configuration in the visual search and the three control tasks. The monkey had to respond respectively to the blue bar (visual search), or to an increase in the luminance of either the fixation point (central control), a part of the background whose location was fixed and predefined by block (fixed peripheral control), or a part of the background occurring randomly at one of the bar positions (random peripheral control). The dotted parts of background are presented only for the sake of comprehension and correspond to potential locations for the change of luminance. The color of the fixation point (red, light grey, dark blue, or light green) indicated the nature of the task. Two- and 4-item configurations were also tested. B, Sets of data obtained during the scanning. In a time series, the visual search was mixed either with the central control, the fixed peripheral control or the random peripheral control. In each case, the visual search condition was adapted to match visually its control (i.e. presence of the corresponding change of luminance in the background at the end of each trial, but ignored by the monkey). The control conditions contained trials with red bars only and trials with blue bars only, in order to exactly match the visual stimulation with that during visual search. Therefore, in each set of data, the visual search and the control were equated for visual input and motor output. One major difference between search and each of the controls was the need to select the target amongst similar competing objects (i.e. bars). Another difference concerned the spatial allocation of attention, which was matched only in the vs/rpc case.

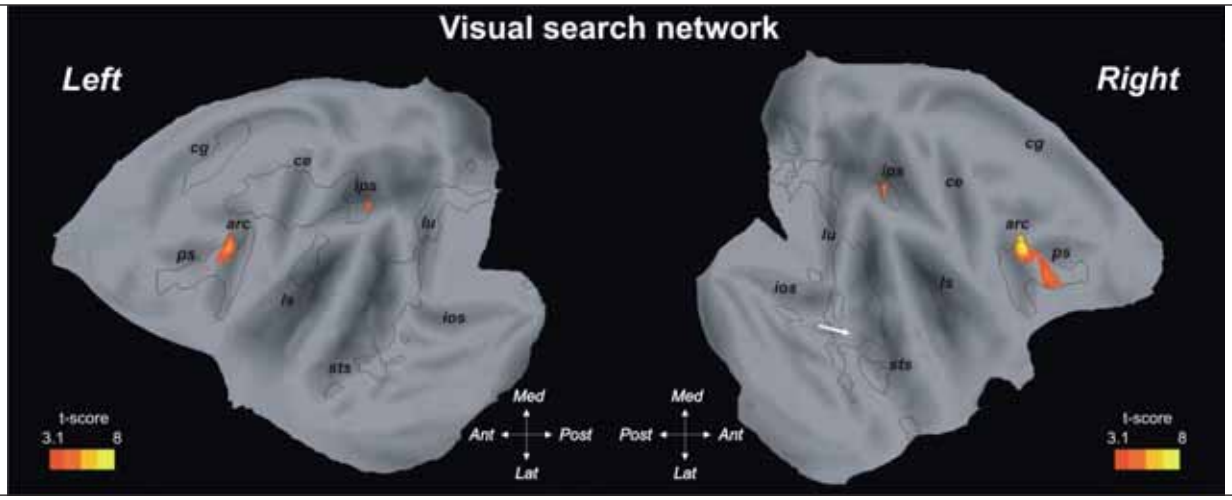

Figure 2: Specific visual search network displayed on flattened representations of the brain. Statistical parametric maps (SPMs) of the conjunction analysis (group of two monkeys) of three contrasts, visual search *vs.* each control task ($p<0.001$ for each individual contrast, uncorrected for multiple comparisons, and masked to display only positive signal changes relative to the fixation baseline) are displayed on the flattened representations of both hemispheres using Caret. The black outlines represent the contours of the general network. The white arrow indicates the right TE activation site. Sulci: *arc* arcuate sulcus, *ce* central sulcus, *ios* inferior occipital sulcus, *ips* intraparietal sulcus, *ls* lateral sulcus, *lu* lunate sulcus, *ps* principal sulcus, *sts* superior temporal sulcus.

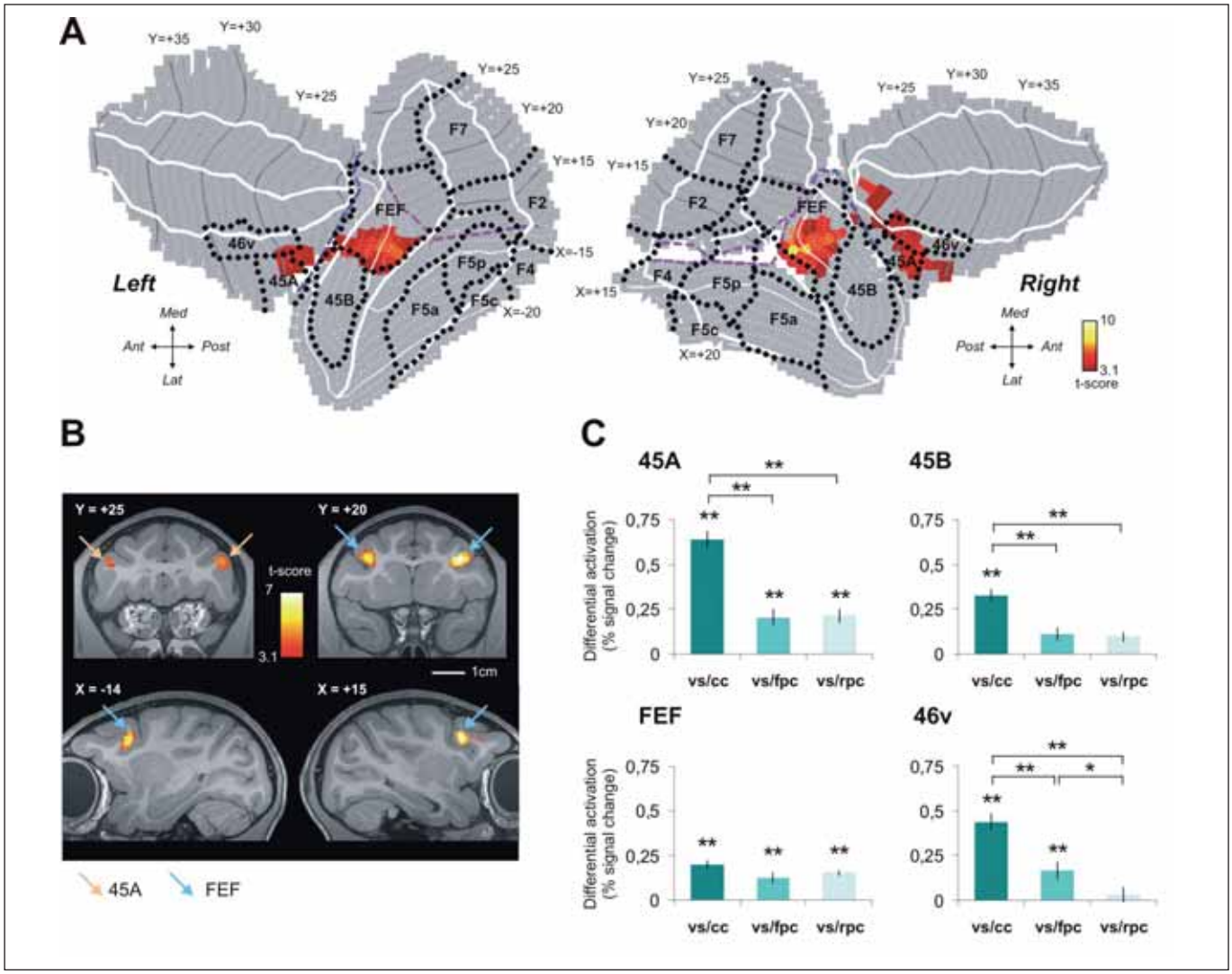

Figure 3: Specific frontal activations in the visual search task. SPMs of the conjunction analysis (group of two monkeys) of the three contrasts (each at p<0.001, uncorrected) visual search *vs.* each control condition, displayed on the flat maps of the principal and arcuate sulci in A and on coronal and sagittal brain sections in B. In A, thick white lines represent the fundus and the two lips of the sulci. The thin white solid and dotted lines represent points with the same X coordinates, the gray ones the same Y coordinates. Thick black dotted lines represent the outlines of the labeled anatomical ROIs. Purple or blue dashed lines indicate the transitions between the three separately computed maps. Activations are masked to display only positive signal changes relative to the fixation baseline (visual search and control *vs.* fixation, p<0.05 uncorrected level). C, ROI analysis. The histograms display the differences in MR signal between the visual search and each of the 3 controls, within the four ROIs (vs/cc: visual search compared to central control; vs/fpc: fixed peripheral control; vs/rdc: random peripheral control) in percent signal change (± sem). *: p<0.05, **: p<0.001, *post-hoc* Bonferoni test with multiple comparisons (number of ROIs); significance of the difference for a given control condition within a region assessed in a 3-way ANOVA with task (n=2), control type (n=3) and region (n=4) as factors (3-way interaction p<0.00001); significance of the difference between types of control within a region assessed with 2-way ANOVAs with task and control type as factors (2-way interactions significant in 45A, B and 46v).

2. Bottom-up Dependent Gating of Frontal Signals in Early Visual Cortex (Leeland Ekstrom, Pieter Roelfsema, Giorgio Bonmassar, John Arsenault)

The frontal eye fields (FEF) are one of several cortical regions thought to modulate sensory inputs. Moreover, several hypotheses suggest that FEF can only modulate early visual areas in the presence of a visual stimulus. To test directly for a bottom-up gating of frontal signals, we microstimulated subregions in FEF of two monkeys and measured the effects throughout the brain with functional magnetic resonance imaging. The activity of higher-order visual areas was strongly modulated by FEF stimulation, independent of visual stimulation. In contrast, FEF stimulation induced a topographically specific pattern of enhancement and suppression in early visual areas only in the presence of a visual stimulus. We conclude that bottom-up activation is needed to enable top-down modulation of early visual cortex.

Contemporary theories propose that feedback signals from areas in frontal and parietal cortex exert control over the processing of incoming visual information. Several models suggest that these signals are gated by bottom-up stimulation. In these models, feedback signals only influence neurons activated by visual input, just as has been observed for attentional effects, which are known to be strongest for neurons well driven by a visual stimulus. No causal evidence exists, however, to support these theories, with the exception of area V4, where feedback effects evoked by stimulation of the FEF are most pronounced for neurons strongly activated by a visual stimulus. To i) test these models of bottom-up dependent gating of frontal signals on a whole brain scale, ii) investigate the impact of increased FEF activity on visually-driven responses throughout occipito-temporal cortex and iii) examine the spatial organization of any observed modulations, we developed a combination of functional magnetic resonance imaging (fMRI) and chronic electrical microstimulation (EM) in awake, behaving monkeys.

If feedback effects are gated by visual stimulation one predicts FEF-EM effects in visual areas separated from the FEF by multiple synapses, in the presence of a visual stimulus. We therefore placed colored, moving gratings in the MFs of the FEF stimulation sites under passive viewing conditions, and measured the fMRI response to EM-only, visual-only (V), and combined visual-EM (VEM) stimulation, relative to a fixation-only condition (i.e. 2x2 factorial design with factors EM and visual stimulation).

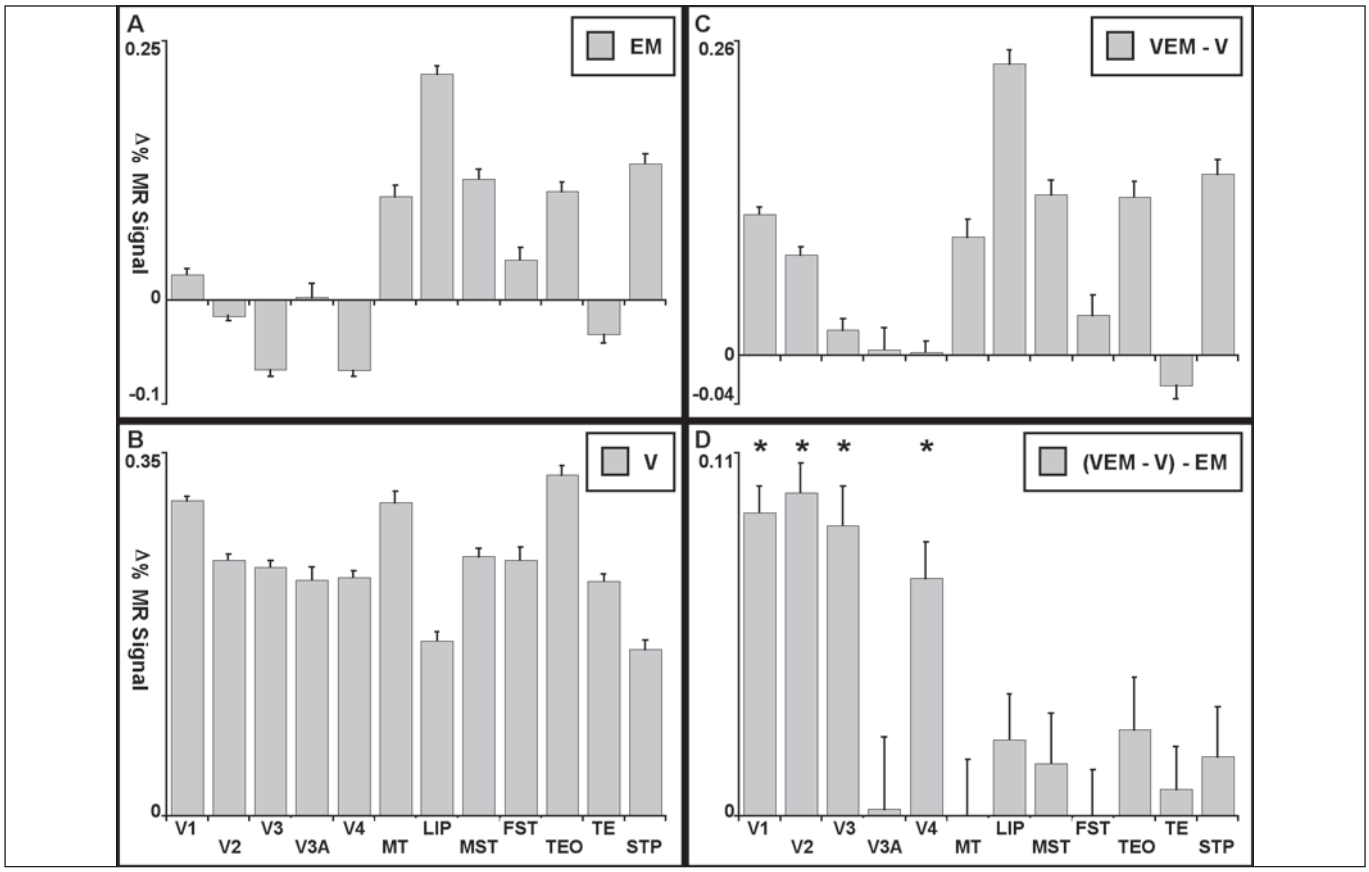

Figure 4 Gating of FEF-EM effects by a visual stimulus. Mean % change in MR signal (MM1 and MM2) for all visually-driven voxels ($p < 0.05$, uncorrected) in 12 visual areas for (A) EM and (B) V epochs, (C) the difference between VEM and V epochs, and (D) the interaction (VEM-V-EM). Error bars denote SEM; * indicates a significant difference between the VEM-V and EM distributions ($p < 10^{-17}$, two-sample, two-tailed t-test), revealing that on average voxels in early visual areas show a larger EM response in the presence of visual stimuli than in the absence. Higher order visual areas show an EM response both with and without visual stimulation.

To investigate the net influence of visual stimulation on FEF-EM effects, we compared EM minus fixation (Fig. 4A) to VEM minus V (Fig. 4C) in all visually-driven voxels for a number of cortical areas; baseline visual activity is shown in Figure 2B. Visually-driven voxels in areas directly connected to the FEF, such as LIP and several areas within the superior temporal sulcus, showed an EM-driven increase in fMRI activity, relatively independent of the presence of a visual stimulus (compare Figs. 4A and 4C). In contrast, visual stimulation unveiled a significant influence of FEF-EM on the activity of early visual areas (V1 and V2). Figure 4D isolates the effect of the visual stimulus on FEF-EM, by subtracting the activity evoked by EM in the absence of a visual stimulus (Fig. 4A) from the activity evoked in the presence of a visual stimulus (Fig. 4C). Visual stimulation enabled the effects of FEF microstimulation to reach early visual areas, including V1, which is not mono-synaptically connected to the FEF.

We have demonstrated spatially-specific, causal interactions between activity in area FEF and many areas of the visual cortex. Signals from frontal cortex activated higher-order areas directly connected to the FEF irrespective of visual stimulation, mimicking attention-driven baseline shifts in activity. In the presence of visual stimulation, modulations were observed at even the earliest levels of visual cortex, including area V1. These effects are likely trans-synaptic, because these early areas do not receive direct connections from the FEF and were only observed when

the neurons were congruently activated by a visual stimulus. One interpretation is that visual stimulation opens feedback pathways closed in the absence of stimuli, allowing these frontal signals to propagate from higher to earlier visual cortical areas. The effect of FEF-EM thus resembles spatial attention, which interacts with visual stimuli in a comparable, multiplicative manner. Our results thereby strengthen past observations that structures involved in generating eye-movements are well-suited to modulate sensory-driven activity in a topographically specific manner.

The voxels most strongly enhanced by FEF-EM were adjacent to those voxels with the strongest visual response, while the strongly driven voxels themselves were unaffected or even suppressed by FEF-EM. These results are in accordance with a recently proposed model for the effects of feedback connections that suggests that feedback and horizontal connections can mediate a contrast-dependent inhibition of a central zone in the next lower area, while still exciting the near surround. That the effects of FEF-EM are most apparent in less optimally driven voxels may also parallel past findings that attentional effects are larger in the presence of competitive distracters that initially reduce neuronal responses in unattended conditions.

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Final 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?

Introduction:

The 5-HT7 receptor (5-HT7R) belongs to the family of G protein-coupled receptors (GPCR's) and is the most recently identified 5-HT receptor. Alternative splicing of the 5-HT7R-mRNA gives rise to 4 different isoforms in rat (a, b, c and e) and 3 in human (a, b and d) [1-3]. The 5-HT7R is involved in diverse physiological processes, such as circadian rhythm, REM ('rapid eye movement') sleep, thermoregulation, learning and memory and endocrine regulation. Malfunctions, associated with this receptor are also numerous, including depression, migraine and cognitive and anxiety disorders [4, 5]. This diversity in (patho)physiological processes probably reflects a complex signaling pathway arising from the 5-HT7R. However, apart from the fact that this receptor is Gs-coupled and able to activate MAP kinase, very little is known about this signaling pathway. In order to better understand this pathway, we decided to search for new intracellular interaction partners of the 5-HT7 receptor. Therefore, we performed a yeast two-hybrid (Y2H) screening with the C-terminal tail of the human 5-HT7aR as bait and an adult-human brain cDNA library as prey. Several potential interaction partners were identified, of which APLP1 (amyloid precursor-like protein 1) was of high interest. This interaction could namely implicate 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 are all type I integral, membrane proteins with a large glycosylated extracellular region, one transmembrane helix and a short C-terminal cytoplasmic tail. Another common characteristic of these proteins is the fact that they are consecutively processed by 2 proteases. First, the extracellular part is cleaved close to the membrane by the α- or β-secretase resulting in the formation of a soluble extracellular domain (sAPPα/β or sAPLPα/β) and an intermediate membrane-bound fragment (C83 and C99, respectively). The latter is then further cleaved by the γ-secretase. Sequential cleavage of APP by the β-secretase and the γ-secretase results in the secretion of the amyloid β-peptide [6-8]. This is the most prominent component found in the brain tissue of patients with Alzheimer's Disease (AD). This disease is characterized by two types of protein aggregates in the brain: the intracellular neurofibrillary tangles (NFT), which consist of hyperphosphorylated tau, and the extracellular amyloid plaques, containing mainly amyloid-β peptide. AD is the most common form of dementia among elderly people [9]. It is a progressive, neurodegenerative disorder, associated with impairments in cognition and memory. The prevalence of AD is 5-10% at the age of 65 and 20-50% at the age of 85. By 2050, the population over 65 years of age will approximately double, which results to over 20 million of patients with AD in the United States and Europe.

Research: current status and prospectives

1. Interaction studies

As mentioned above, we identified APLP1 as an interaction partner of the 5-HT7aR by means of a Y2H screening. To confirm this interaction in a more physiologically relevant cell system, we performed a co-immunoprecipitation (CoIP) experiment between APLP1 and the 5-HT7aR in COS cells after transient transfection. Not only could we detect a clear interaction of APLP1 with the 5-HT7aR, but we also found interaction with the b and d isoform of this receptor. In a similar way we could demonstrate interaction between APLP2 and APP and all three 5-HT7R isoforms. Interaction of APLP1 with the 5-HT7 receptors could also be confirmed in a neuronal cell line, namely HT22 (a mouse hippocampal cell line) (Figure 1).

In order to verify that the observed interaction is not just an artefact due to transient overexpression of both proteins, we also performed a CoIP in CHO cells, which stably express APP (gift from Drs. D. Walsh and D. Selkoe, Department of Neurology, Harvard Medical School and Center for Neurologic Disease, Boston, Massachussetts). After transient transfection of these cells with the 5-HT7aR and subsequent IP of the latter, we could observe CoIP of the stably expressed APP.

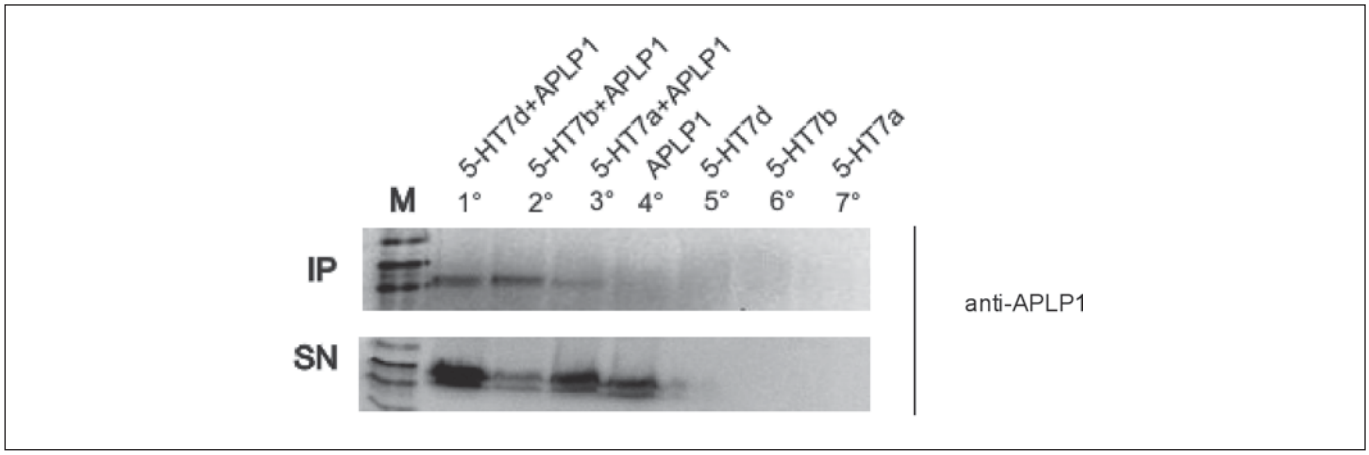

Fig. 1: Co-immunoprecipitation of APLP1 with the 5-HT7R isoforms in HT22 cells.
HT22 cells were transiently transfected with the cmyc-tagged 5-HT7R isoforms and APLP1, alone or in combination. Immunoprecipitation was performed with a mouse antibody against the cmyc-tag
The immunoprecipitated proteins and the input lysates were visualized with anti-APLP1(CT11).

As both the 'APP proteins' and the 5-HT7 receptor are transmembrane proteins, the possibility exists that the coIP of these two proteins is not a consequence of direct interaction, but merely results from the fact that they are both firmly attached to the membrane. To exclude this possibility, we further examined the interaction between these proteins *in vitro,* namely by means of a GST (glutathione-S-transferase) pulldown. After specific precipitation of the C-terminal tail of the 5-HT7(a) receptor, fused to GST (GST-CT(5-HT7aR)) with glutathione beads, we observed co-precipitation of all 3 'APP proteins' (data not shown). Thus, we can conclude that all 'APP proteins' directly interact with all three 5-HT7 receptor isoforms.

In order to determine the domains involved in the interaction between APLP1 and the 5-HT7 receptor, we performed several CoIP experiments with deletion mutants of both proteins. As we

identified APLP1 as an interaction partner of the intracellular C-terminal tail of the receptor, we wanted to know if two intracellular, C-terminal fragments of APLP1 (C51 and C60, provided by Dr. D. Walsh) also co-immunoprecipitate with the receptor. However, the C51 and C60 fragment did not co-immunoprecipitate with the receptor, which is probably due to a mislocation of these fragments. In contrast to wild type APLP1, the C51 and C61 fragments are not attached to the membrane, where the receptor is localized. To avoid this topological problem, we will perform an *in vitro* GST-pulldown experiment using five C-terminal APLP1-deletion mutants, kindly provided by Dr. Weber, University Hospital Schleswig-Holstein, Kiel, Germany, with GST-CT(5-HT7aR).

Finally, we also performed a CoIP of wild type APLP1 with several deletion mutants of the 5-HT7(a) receptor. These lack the C-terminal tail (ΔCT and 7TM), the third intracellular loop (ΔIC3) or both (ΔCTΔIC3 and 5TM). In contrast to the ΔCT mutant, which lacks most part of the C-terminal tail, the 7TM deletion mutant lacks the C-terminal tail completely. Similarly, the 5TM mutant lacks the complete CT and IC3, whereas the ΔCTΔIC3 mutant lacks most part of these intracellular domains. We observed that all these 5-HT7aR deletion mutants still are able to interact with APLP1 (Figure 2). This indicates that the presence of the CT or IC3 is not necessary for this interaction and thus that also other domains are involved in this interaction, which could be the IC1, IC2 or even transmembrane domains of the receptor.

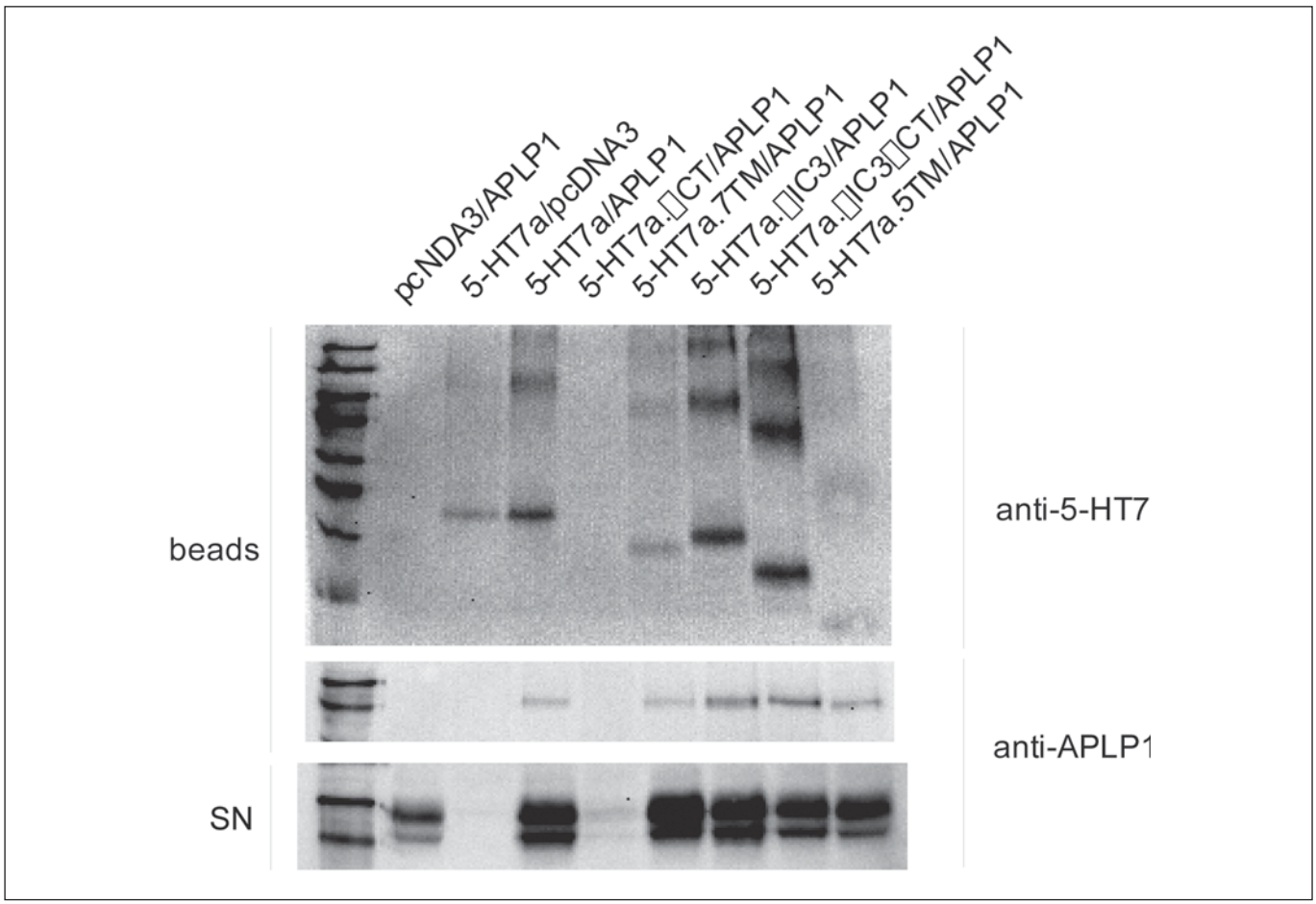

Fig. 2: Co-immunoprecipitation of APLP1 with several deletion mutants of the 5-HT7aR in HT22 cells.
HT22 cells were transiently transfected with wild type cmyc.5-HT7aR and several cmyc-tagged deletion mutants of this receptor, together with APLP1 or APLP1 alone. Immunoprecipitation was performed with a mouse antibody against the cmyc-tag. The immunoprecipitated proteins were visualized with anti-APLP1 (CT11) and with anti-5-HT7 (Biogenes) and the input lysates with anti-APLP1(CT11).

It must be noted that, although we consistently detect two forms of APP/APLP, namely a mature, fully glycosylated form and an immature, partially glycosylated form, we only detected CoIP of the smaller, immature form. This suggests that the interaction does not take place at the plasma membrane, but at the membrane of the endoplasmatic reticulum (ER). This also explains why the endogenous, fully glycosylated APP, observed in both COS and HT22 cells, does not co-immunoprecipitate with the receptor (Figure 3). In order to verify this, we will perform a CoIP experiment both at the plasma membrane and at the ER in future experiments.

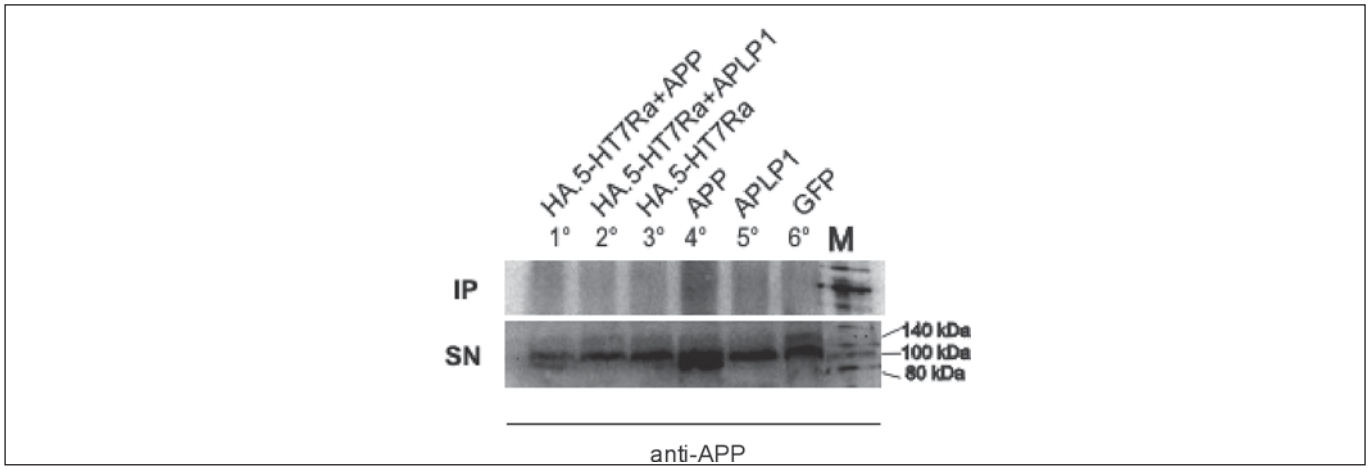

Fig. 3: Endogenous APP does not co-immunoprecipitate with the 5-HT7aR in HT22 cells.

2. Co-localisation studies

It has been shown that APP plays a role in axonal transport of vesicles. Its C-terminal domain interacts with the kinesin light chain and in this way links the transport vesicle with the kinesin motor complex [10]. Secondly, Weber et al. have recently shown that APLP1 alters the localization of the α2A-adrenergic receptor [11]. Therefore we would like to investigate whether APP or APLP1/2 have an influence on receptor localization or transport by means of immunofluorescence. We could already demonstrate a substantial degree of co-localisation between APP and APLP1 and the 5-HT7(a) receptor after transient transfection of COS7 and HT22 cells. It is our aim to further expand these co-localisation studies with endogenous APP, APLP1/2 and 5-HT7 receptor. Therefore we are currently screening several neuronal cell lines for endogenous expression of these proteins (see section 4).

3. APP/APLP processing and secretion

Stimulation of the 5-HT4, 5-HT2A and 5-HT2C receptors leads to an increased secretion of non-amyloidogenic soluble APP (sAPPα) [12-14]. This not only prevents the formation of Aβ, but also gives rise to the neurotrophic sAPPα and thus can be beneficial for the treatment of AD. This is in agreement with a recent study, which shows that activation of the 5-HT4 receptor inhibits secretion of Aβ and increases neuronal survival [15]. Therefore, we investigated if stimulation of the 5-HT7 receptor also leads to an increased secretion of APP or APLP1/2. If so, we will determine the identity of the secreted product as amyloidogenic or non-amyloidogenic. As it has been shown that 5-HT4-mediated secretion of APP as well as 5-HT7-mediated activation of Erk are both Epac-dependent, we will also study the involvement of the latter in 5-HT7-mediated secretion of APP

or APLP1/2 [16, 17]. Our results suggest that co-expression of the 5-HT7(a) receptor has rather a negative effect on the secretion of APLP1 (Figure 4). However, further experiments are necessary to confirm this and in order to obtain more physiological relevant data, we will study the influence of the 5-HT7 receptor on secretion of the APP proteins endogenously in a neuronal cell line. As mentioned above, we are currently screening several neuronal cell lines on endogenous expression of the APP proteins and the 5-HT7 receptor (see section 4).

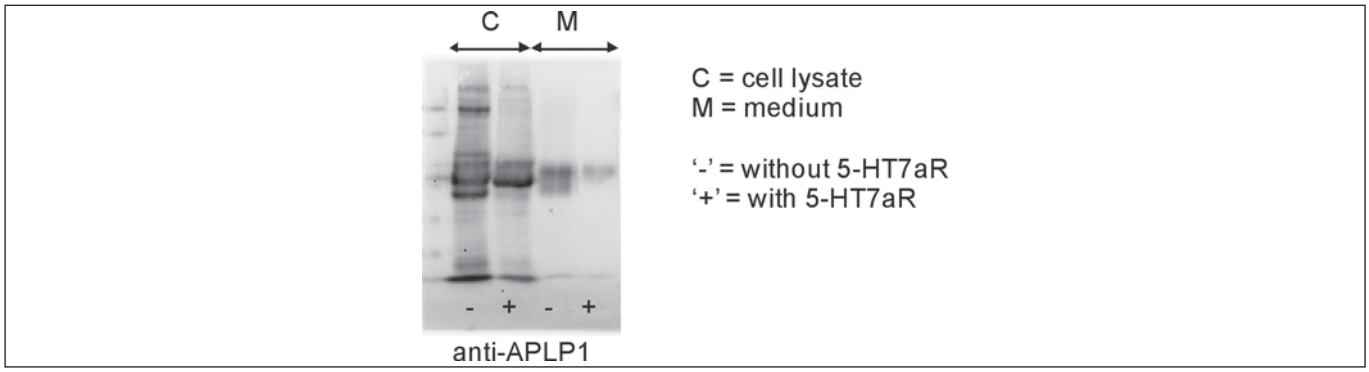

Fig. 4: Influence of the 5-HT7a receptor on secretion of APLP1 in HEK293T cells.
HEK293T cells were transiently transfected with APLP1 with or without the 5-HT7a receptor. Cell lysates and conditioned media were analysed with anti-APLP1(NT).

4. Endogenous expression of the APP proteins and the 5-HT7 receptor

Several immortalized neuronal cell lines are available in the lab and are currently being screened for endogenous expression of the APP proteins and the 5-HT7 receptor. By means of Western analysis we were able to detect endogenous APP and APLP2 in all cell lines tested so far (Figure 5). Nevertheless, the expression levels differ significantly amongst these cell lines.

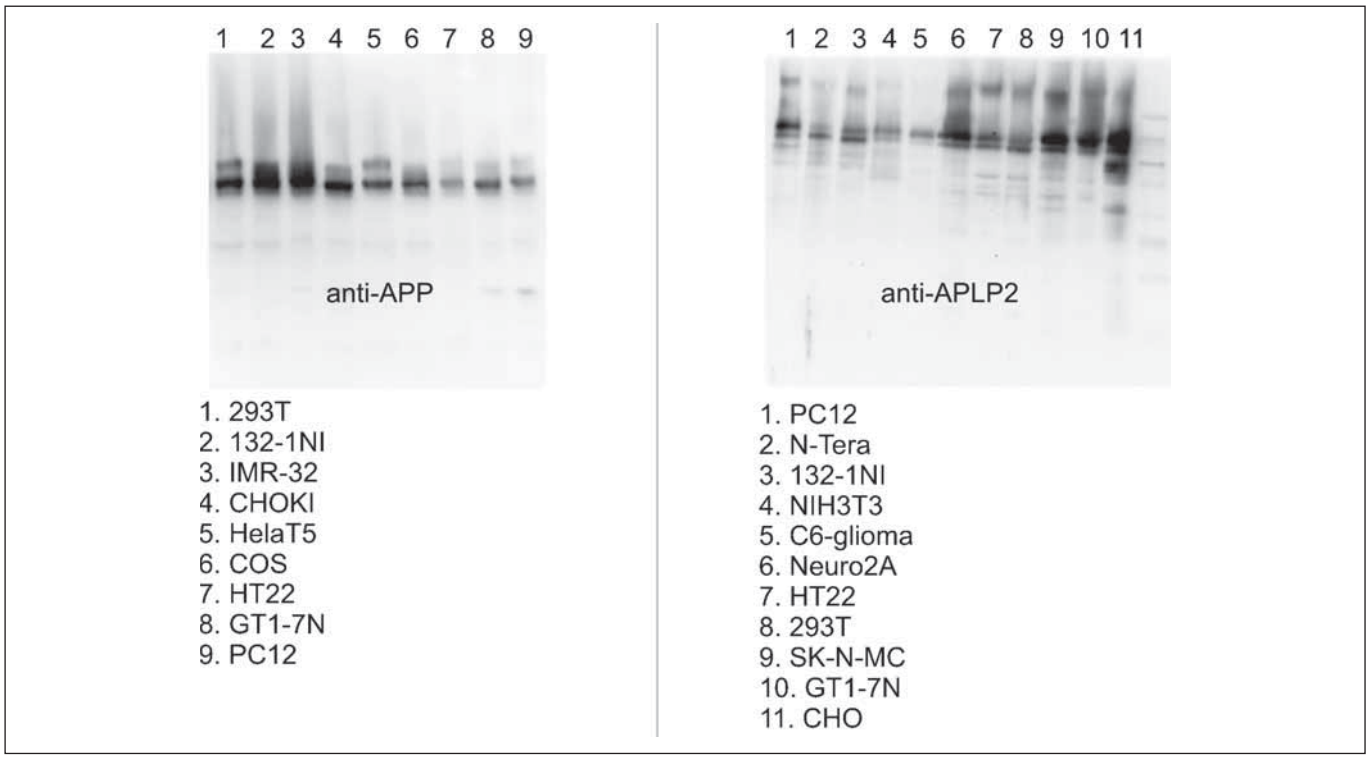

Fig. 5: Endogenous expression of APP and APLP2 in several immortalized neuronal cell lines.

For a number of neuronal cell lines, our RT-PCR data indicate the presence of 5-HT7 mRNA (data not shown). In order to detect the 5-HT7 receptor on the protein level, we tried out several commercial antibodies, but never succeeded in detecting endogenous 5-HT7 receptor. Therefore, we decided to generate our own antibody, which turned out to show a higher affinity for overexpressed 5-HT7 receptor than the commercial ones. Using this antibody we were able to detect endogenous 5-HT7 receptor in a few cell lines, namely GT1-7N, HT22 and 132-NI. We observed a band at about 80 kDA, of which we assume that it corresponds to the 5-HT7 receptor for the following reasons. First, the band disappeared after pre-incubation of the antibody with the specific 5-HT7R peptide, to which the antibody was raised (Figure 6). Second, the intensity of this band increased after treatment with the specific 5-HT7-antagonist, SB269970, as expected for a 5-HT7 receptor band. Finally, the endogenous receptor was detected at the same molecular weight as overexpressed 5-HT7 receptor in HT22 cells. All together, these data strongly indicate that the observed band represents the 5-HT7 receptor. As Western blot analysis reveals a band higher than the molecular weight of the receptor (45 kDa), we suspect that the receptor is glycosylated or forms dimers in these cell lines. Endogenous expression of the receptor will also be checked in the other available neuronal cell lines and these results will be combined with the APP/APLP expression data. Finally, one cell line will be selected to start the *in vivo* studies.

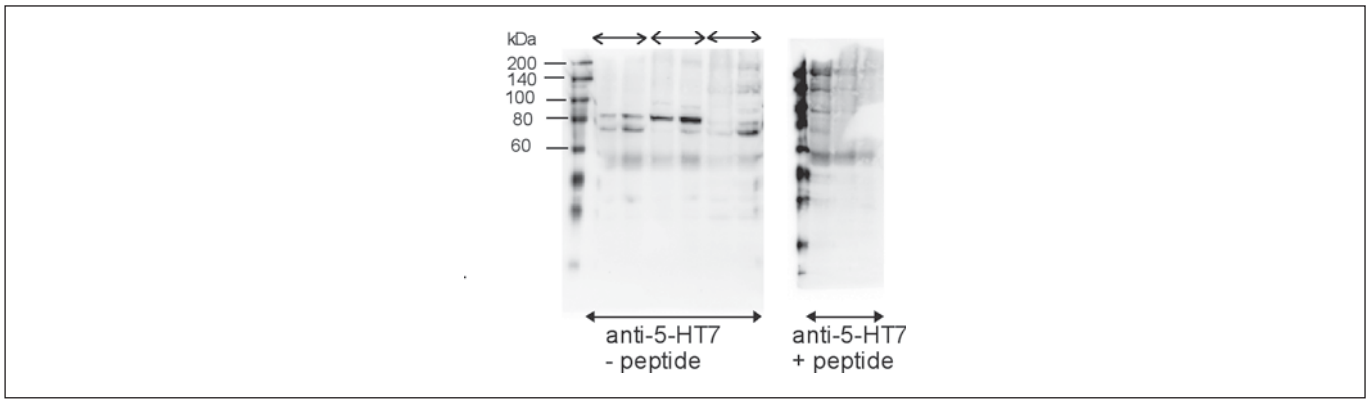

Fig. 6: Endogenous expression of the 5-HT7 receptor in several immortalized neuronal cell lines.

Conclusions:

1. Interaction could be confirmed between APP, APLP1 and APLP2 and the three 5-HT7R isoforms both by CoIP and GST-pulldown.
2. Not only the C-terminal tail and third intracellular loop of the 5-HT7 receptor are involved in the interaction with the 'APP proteins', but also other intracellular and/or transmembrane domains of this receptor.
3. Only the immature form of the 'APP proteins' interacts with the 5-HT7 receptor, not the mature form.
4. A substantial degree of colocalisation could be detected between APP and APLP1 and the 5-HT7 receptor in COS cells and HT22 cells by immunofluorescence.
5. Co-expression of the 5-HT7 receptor leads to a significant decrease in the secretion of APLP1 in HEK293T cells.

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Coding of action categories in primate cortex.

The main topic of our project proposal concerned the neural mechanisms of visual action categorization in temporal cortical neurons. 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 rostral Superior Temporal Sulcus (STS) in the temporal lobe, the parietal cortex, and frontal areas are important for action recognition. We examined the analysis of dynamic action stimuli by temporal cortical neurons, including the rostral STS, of macaque monkeys. This study was performed in fixating monkeys, which allowed a fuller exploration of the functional response properties of the neurons, using a wider range of stimuli than what is possible in monkeys trained to categorize particular images.

In addition to this study of the analysis of visual dynamic action stimuli, we studied the effect of stimulus history on the responses of inferior temporal (IT) neurons. Part of the selectivity for dynamic action sequences might be due to the effect of preceding stimuli in a sequence to the response to the next stimulus of that sequence. One well known effect of stimulus history in visual cortex is adaptation, being the reduction in the response upon repetition of a stimulus. In the course of the project we have studied the stimulus selectivity of this adaptation effect in macaque temporal cortex.

A third related topic we studied was the effect of categorization learning on the shape tuning of IT neurons. In this study we employed static instead of dynamic stimuli. We did not employ dynamic action stimuli in this categorization learning study since we wished to understand in more detail the coding of the latter dynamic stimuli by IT neurons, before investigating the effect of categorization training on the coding of dynamic stimuli.
Below I will discuss the related research topics and the obtained results in detail.

1. Analysis of visual action stimuli by temporal cortical neurons.

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. The parameterized stimulus set was developed in collaboration with Dr. F. Pollick (Univ. Glasgow, UK). We have examined the coding of a parameterized set of dynamic images of simple actions by macaque inferior temporal neurons. We used arm actions like knocking, lifting and throwing. The action images were rendered as stick figures. The objects of the goal-directed actions were not rendered. The experimental design that we employed to test the responses of visual neurons had several novel features. First, we used a parameterized set of actions that consisted of three-way blends of 3 prototypical arm actions. 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.

Second, we compared the responses of the neurons to the dynamic action stimuli to static snapshots of the action sequences so that we could assess the contribution of form versus motion information to the responses of the full actions. In addition, we measured the neuronal responses to snapshots translating in two opposite directions, to assess direction and motion selectivity. Third, since the actions were restricted to one limb we could systematically reduce the full (stick-) body displays to more simple displays consisting of e.g. the moving limb only or even the end-effector (wrist) dot only. This enabled us to determine the contribution of the body (stick-) configuration to the responses of the neurons to the full action stimuli. Additional tests included presentations of the actions at different spatial positions and a reversal of the action sequences. We have recorded mainly in the dorsal and ventral bank of the rostral Superior Temporal Sulcus (rostral STS; visual areas STP and TE), and the lateral convexity of the inferior temporal cortex.

Analysis of the single cell recordings in two animals (n= 240 neurons) indicated that rostral STS neurons respond selectively to temporal segments of the action sequences, but not to the whole action as such. Nonetheless, as a population, the neurons represented the similarity among the different actions. This was shown by performing a non-linear multidimensional scaling (ISOMAP) of the pairwise differences between the neural responses to the different stimuli: the low-dimensional ISOMAP configuration based on the neural responses corresponded at the ordinal level to that of the parametric configuration. Thus, as we have shown before for static shapes (Op de Beeck et al., Nature Neurosci., 2001), temporal cortical neurons can represent the similarity between dynamic action stimuli. Such representation of the similarity of action stimuli can be used as input for an action categorization process. Interestingly, the average population responses were significantly larger for the prototypical actions than for the blends. This bias for the extremities of the parametric configuration has also been observed for static shapes (Kayaert et al., Eur. J Neurosci., 2005) and face stimuli in IT (Leopold et al., Nature, 2006).

Based on our different tests, we could distinguish two kinds of neuronal selectivities. First, neurons, mainly in the ventral bank of the rostral STS and the lateral convexity, responded equally well to the action stimuli as to presentations of static snapshots and responded less to the moving arm alone than to whole body configuration. These neurons clearly responded to form information. These "snapshot neurons" can contribute to action coding by analyzing the form of the actor as postulated in several computational models of action recognition (Lange et al., J. Vision, 2006; Giese and Poggio, Nature Reviews: Neurosci., 2003). Second, other neurons, mainly in the dorsal bank of the rostral STS responded much less to static snapshots than to the dynamic action images, clearly responding to motion information. Most of these "motion neurons" responded equally well to the whole body configuration as to the moving arm alone. In fact, further testing showed that most of the "motion neurons" responded at least equally well to the motion of the hand dot alone. In depth analysis of the data showed that the representation of the action space was less faithful for the "snapshot" compared to the "motion neurons", which likely follows from the observed greater action selectivity for the "motion" compared to the "snapshot neurons".

Both groups of neurons showed strong modulation of their responses during the course of the actions. The average response of the "motion neurons" during the action correlated with speed

of the arm movement in the visual display. However, mere speed did not explain the action selectivity of the "motion neurons" since (1) the neural representation of the action space was 2D (triangular configuration mimicking the parametric configuration) and not 1D (speed is a one-dimensional parameter), and (2) reversal of the action sequence, and thus speed profile, did not reverse the response profile. This data suggest that the "motion neurons" code for the kinematics or movement trajectory of the action but not the action per se. We were able to show that a small population of such "motion neurons" (n=50) provided a highly faithful representation of the similarity among visual action sequences, and this is information is highly useful for action coding by downstream neurons.

The response modulation during the course of the action in the "snapshot neurons" was mainly related to their snapshot selectivity: these neurons responded to segments of the action that contained snapshots the neuron is selective for (effective snapshot). However, the mere presence of the effective snapshot was not sufficient to have a response in "snapshot neurons", since about 1/3 of these neurons did not respond to actions in which the effective snapshot was present. This can be due to the short duration of the effective snapshot in those actions or a sensitivity to the temporal context or stimulus history (see below).

In conclusion, these single cell data suggest that upper-bank/fundus STS "motion neurons" code for visual kinematics while the lower-bank STS "snapshot neurons" code for form/posture, and that both can contribute to action recognition, in agreement with computation models of action recognition. In particular, the computational model by Giese and Poggio (Nature Reviews Neuroscience, 2003) proposes 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. 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 snippets of the action sequence and finally integration of the temporal sequence of the different that define the action. Our finding of "snapshot" and "motion neurons" in temporal cortex, responding to visual actions, agrees with this proposal.

2. Mechanisms of adaptation in IT.

The reasons behind our interest in neural adaptation, the reduction in activity upon repetition of a stimulus, are twofold: (1) understanding the influence of a stimulus on the response to a successive stimulus of a stimulus sequence, which is relevant for understanding the coding of action sequences (see above), and (2) determining the validity of the fMRI-adaptation technique. fMRI adaptation is becoming a popular tool to infer the tuning for stimulus parameters of neurons in humans. 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 (B-A sequence) than when repeating A (A-A sequence), but adapting to C will produce no response reduction for

A (C-A sequence). We have tested this assumption by single cell recordings in the anterior 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. 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 or only little decrement in response to a successively shown stimulus to which the neuron did respond (CA sequence). The weaker than expected adaptation for a sequence of two different stimuli to which the neuron responded was found in two different adaptation paradigms and for different stimulus durations and interstimulus intervals.

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 likely reflects local, synaptic changes or adaptation of input neurons. Thus, the degree of transfer of adaptation from one stimulus to another may not only depend on how strongly the neuron responds to the stimuli, but on the number of common synapses or afferents for the two stimuli.

3. Effect of categorization learning on shape tuning in IT.

Several studies, starting with Vogels (Eur. J. Neurosci., 1999), suggest that single IT neurons do not represent categories of static images as such but instead show selectivity for exemplars of the same category (within-category selectivity) as well as for exemplars of different categories (between-category selectivity). Still, it is possible that the tuning of IT neurons is influenced by categorization learning. Indeed, it has been suggested that the responses of IT neurons reflect the perceptual similarity among stimuli (Kayaert et al., J. Neurosci., 2003; Op de Beeck et al., Nature Neurosci., 2001) and the latter could be affected by categorization: stimuli are perceptually more distinct along stimulus dimensions relevant for categorization than along dimensions irrelevant for categorization. Thus it is possible that IT neurons are more narrowly tuned along categorization-relevant stimulus dimensions than along irrelevant dimensions and/or that IT neurons show a greater selectivity for exemplars belonging to different learned categories than for exemplars belonging to the same categories.

Previous studies have reported diverse effects of the categorization of images on the selectivity of neurons in macaque IT cortex for these images. Cells in IT cortex showed very weak category effects (Freedman et al., J. Neurosci., 2003): responses to shapes that belong to the same learned category were slightly more similar than responses to shapes that belong to different learned categories. However, this small effect could have been due to physical differences amongst the stimuli instead of differences in category membership. Another study reported that the IT representation of features that were diagnostic for distinguishing between two learned categories

of stimuli was enhanced compared to the representation of non-diagnostic features (Sigala et al., Nature, 2002). However, this relatively large effect of categorization relevant feature selectivity can be due to mere stimulus selectivity, unrelated to categorization learning, since no pre-training measurements of selectivity were obtained and the diagnostic features were identical in both animals. Neither such expansion of the relevant dimension, nor any other metric change of IT neural representation was found in a study where different categorization rules were applied (Op de Beeck et al., Nature Neurosci., 2001).

Given these diverse effects of categorization learning on IT tuning in the previous studies and the methodological concerns that preclude valid conclusions from these studies, we re-visited the effect of categorization learning on IT shape tuning. In this new study we controlled for pre-learned stimulus selectivity effects versus learned category-related effects in two ways: (1) we recorded the selectivity of IT neurons before and after categorization learning, and, (2) the relevant categorization dimensions were counterbalanced across animals. In our study, 4 sets of 2D shapes were created combining the shape dimensions "aspect ratio" and "curvature", which were perceptual separable as tested beforehand in humans. A psychophysical study in humans (Ons et al., ECVP, 2006) showed that learning to categorize these shapes resulted in an improved discriminability for the relevant shape dimension. In a first part of our experiment, we recorded the responses of single IT neurons while presenting all shapes randomly intermixed for 100ms/image in a Rapid Serial Visual Presentation (RSVP) paradigm while the monkeys were passively fixating. In an earlier study, we validated this RSVP method as a reliable tool to measure the stimulus selectivity of IT neurons (De Baene et al., J. Neurophysiology, 2007). After the pre-training RSVP measurements, we trained the 2 monkeys to group stimuli into 2 categories with curvature and aspect ratio as the relevant dimension for the first and second monkey, respectively. After training, we recorded the responses of single IT neurons to the shapes while the monkeys were categorizing the images.

Analysis of the data provided no convincing evidence for enhanced tuning along the relevant compared to the irrelevant categorization dimension. Although using the same selectivity metric as Sigala and Logothetis, we observed a greater selectivity along the relevant compared to the irrelevant dimension, this effect disappeared when using another selectivity metric that took into account response variability and did not normalize the responses. However, the categorization training shifted the ratio between within-category neural response similarities and between-category response similarities to a larger value for the relevant dimension. However, this effect was small, but significant. These results suggest that shape categorization learning can induce minor changes in the shape tuning of IT neurons. Thus it is likely that the bulk of the categorization learning behavioral effect is determined by neural changes that occur downstream from IT, that is in regions that read out the IT activity.

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