Verslag – Rapport – Report

2009

G.S.K.E. – F.M.R.E. – Q.E.M.F.

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Geneeskundige Stichting Koningin Elisabeth

Fondation Médicale Reine Elisabeth

Queen Elisabeth Medical Foundation

Geneeskundige Stichting Koningin Elisabeth
2009
Inleiding verslag activiteiten van de GSKE – FMRE

Het jaar 2009 is een overgangsjaar waarin de universitaire teams, die van kredieten genieten, actief hun onderzoeken verder zetten.

De zitting van 4 juni heeft in het Koninklijk Paleis de prijswinnaars van de prijzen UCB, Solvay, Baron Van Gysel de Meise, CBC en Monique Brauns verenigd. Talrijk waren degenen die deze briljante onderzoekers zijn komen toejuichen.

De driejaarlijkse prijs UCB met een waarde van 100.000 euro werd toegekend aan professor Rik VandenBerghe, neuroloog aan de K.U.Leuven, voor zijn project: “functional reorganisation of the language system in probable Alzheimer’s disease, primary progressive aphasia and cognitive aging.”

De prijs werd hem door Prinses Astrid overhandigd, dit in aanwezigheid van Mevrouw Löw-Friedrich, verantwoordelijke voor de ontwikkeling van het onderzoek in de UCB Groep.

De jaarlijkse prijs Solvay, van 25.000 euro, werd overhandigd aan professor Pierre Vanderhaeghen van de ULB, voor de voortzetting van zijn onderzoeken in het kader van zijn project, dat in 2008 werd voorgelegd. De prijs Baron Van Gysel de Meise werd overhandigd aan professor Peter Carmeliet voor de vooruitgang van zijn onderzoek op gebied van de vascularisatie. De prijs CBC werd overhandigd aan Professor Pierre Maquet voor zijn onderzoek op gebied van de slaap en tenslotte werd de prijs Monique Brauns toegekend aan Professor André Goffinet voor de vooruitgang van zijn onderzoek in het domein van de neuronale migraties van de cerebrale cortex.

Deze zitting heeft het mogelijk gemaakt om in het Koninklijk Paleis een belangrijk aantal persoonlijkheden uit de wetenschappelijke, politieke en diplomatieke wereld en de leden van de bekroonde onderzoekteams te ontvangen. Bij deze gelegenheid heeft Prinses Astrid een zeer belangrijke redevoering gehouden over de situatie van de stichting en over de toekomstvisies. Deze zitting heeft het eveneens mogelijk gemaakt om beter te begrijpen wat “de geest van de Stichting” betekent, een gevoel deel uit te maken van een grote familie van onderzoekers die zich allen toeleggen op het onderzoek om het welzijn en de gezondheid voor iedereen te verbeteren. Een monnikenwerk om beter de mechanismen te ontrafelen, die aan de basis liggen van de verschillende neurologische ziektes.

Dit jaar nog heeft Prinses Astrid het laboratorium van neurobiologie en gen therapie van Professor Veerle Baekelandt aan de K.U.Leuven bezocht. Eens te meer was dit bezoek een groot succes. Zij heeft zich dat gerealiseerd tijdens Haar bezoek in gezelschap van de gulle schenker van de prijs “Burggravin Valine de Spoelberch”: Mevrouw Elisabeth Speeckaert met haar echtgenoot Mijnheer Eric Speeckaert.

Het hoge niveau van het onderzoek en de kwaliteit van de resultaten werden voorgesteld in aanwezigheid van de hoogste instanties van de Universiteit en van de stad Leuven. De bezoekers waren erg onder de indruk van de serene sfeer die er heerste in het team van de onderzoekers.

In de loop van dit jaar hebben wij een uitgebreide briewisseling gevoerd met de verantwoordelijken van de “Stichting Marc Hurard”. Hun raad van bestuur heeft besloten om het geheel van de overblijvende middelen over te hevelen naar de Geneeskundige Stichting Koningin Elisabeth. Deze onderhandelingen zullen in 2010 voortgezet worden.

Uiteindelijk staan wij erop om H.K.H. Prinses Astrid, de leden van de raad van bestuur en de leden van het wetenschappelijk Comité te bedanken voor hun permanente steun en hun hulp die zij geven aan de Stichting en haar activiteiten.

Namens alle onderzoekers, dank ik hen hiervoor zeer oprecht.

Prof. em. dr. Baron de Barsy,
wetenschappelijk directeur
Brussel, 31 december 2009

Fondation Médicale Reine Elisabeth
2009
Introduction rapport d'activités de la FMRE - GSKE

L'année 2009 est une année de transition au cours de laquelle les équipes universitaires, bénéficiant de crédits, poursuivent activement leurs recherches.

La séance du 4 juin a réuni au Palais Royal les lauréats des prix UCB, Solvay, Baron van Gysel de Meise, CBC et Monique Brauns. Nombreux sont ceux qui sont venus applaudir ces brillants chercheurs.

Le prix trisannuel UCB d'une valeur de 100.000 euros a été attribué au professeur Rik VandenBergch, neurologue à la K.U.Leuven pour son projet intitulé : functional reorganisation of the language system in probable Alzheimer's disease, primary progressive aphasia and cognitive aging.

Le prix lui a été remis par la Princesse Astrid en présence de Madame Löw-Friedrich, responsable du développement de la recherche à la société UCB.

Le prix Solvay annuel, de 25.000 euros, a été remis au professeur Pierre Vanderhaeghen de l'ULB pour la poursuite très fructueuse de ses recherches dans le cadre du projet soumis en 2008 et le prix Baron van Gysel de Meise a été remis au professeur Peter Carmeliet pour l'avancement de ses recherches dans le domaine de la vascularisation. Le prix CBC au Professeur Pierre Maquet pour ses recherches dans le domaine du sommeil et enfin le prix Monique BRAUNS a été attribué au Professeur André Goffinet pour l'avancement de ses recherches dans le domaine des migrations neuronales du cortex cérébral.

Cette réunion a permis de recevoir au Palais Royal un nombre important de personnalités du monde scientifique, politique et diplomatique et des membres des équipes de recherche primées. A cette occasion, le Princesse Astrid a prononcé un discours très important sur la situation de la fondation et sur les visions d'avenir. Cette réunion a également permis de mieux saisir ce qui fait "l'esprit de la Fondation'', un sentiment d'appartenance à une grande famille de chercheurs qui œuvrent tous à la recherche du bien être et de la santé pour tous, par un travail de fourmis destiné à mieux comprendre les mécanismes qui sont à la base des différents maladies neurologiques.

Cette année encore la Princesse Astrid a pu visiter le laboratoire de neurobiologie et thérapie génique du Professeur Veerle Baekelandt à la K.U.Leuven. Une fois de plus cette visite a été un grand succès. Elle a pu se réaliser en présence du généreux donateur du prix "Vicomtesse Valine de Spoelberch", Madame Elisabeth Speeckaert accompagnée de son mari Monsieur Eric Speeckaert. Le haut niveau de recherche et la qualité des résultats présentés en présence des plus hautes instances de l'Université et la ville de Leuven, associé à une ambiance de sérénité et de fraternité parmi l'équipe des chercheurs, a fortement marqué les invités de ce jour.

Au cours de cette année, nous avons eu plusieurs échanges épistolaires avec les responsables de la Fondation Marc Hurard, dont le conseil d'administration a décidé de transférer l'entièreté des fonds résiduels à la Fondation Médicale Reine Elisabeth. Ces tractations se poursuivront en 2010.

Enfin, nous tenons à remercier chaleureusement SAR la Princesse Astrid, les membres du conseil d'administration et les membres du comité de direction pour leurs encouragements constants, leurs aides sans compter et le soutien permanent qu'ils accordent à la Fondation et à ses activités.

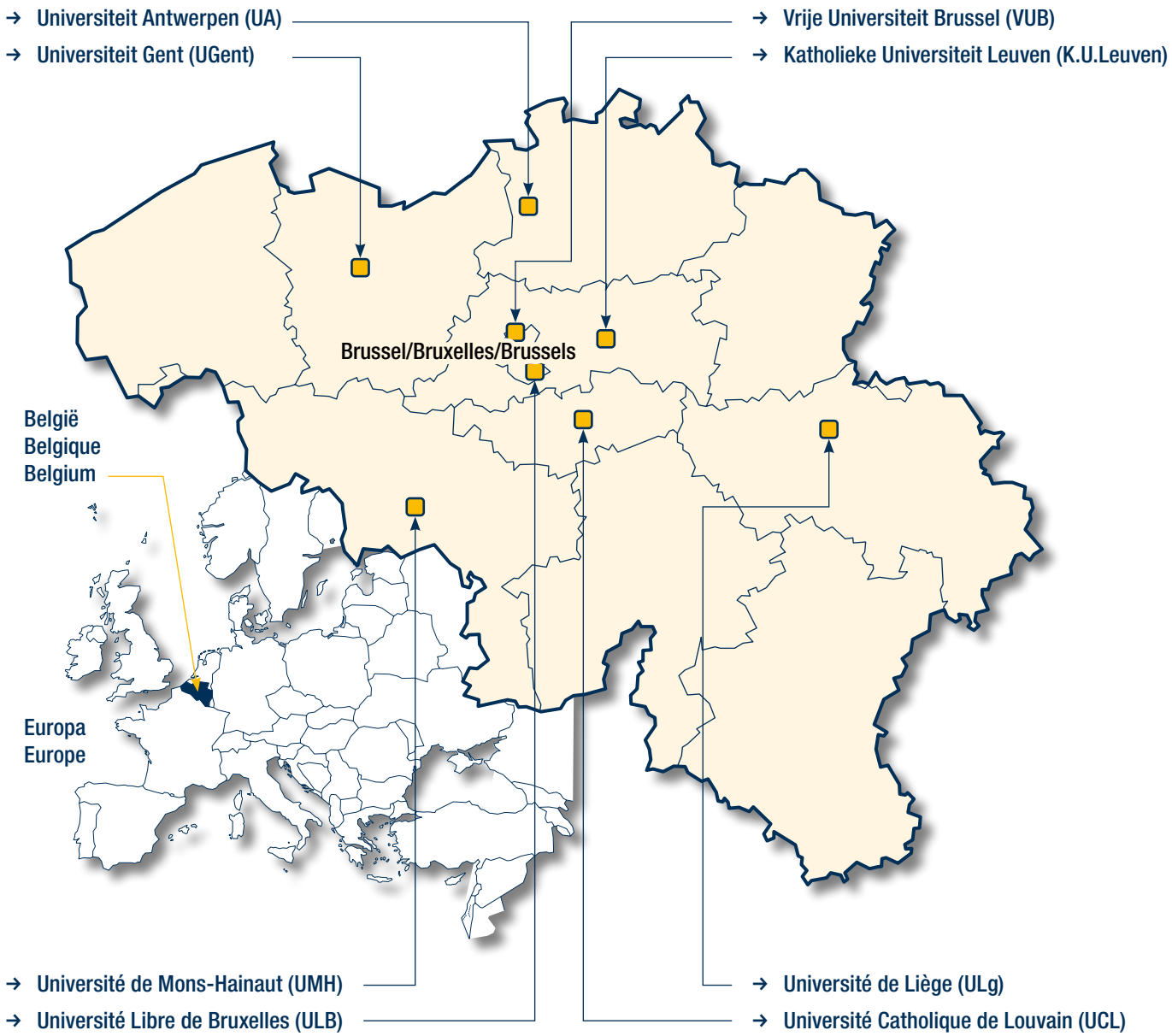
Au nom des chercheurs, qu'ils soient remerciés ici très sincèrement.

Prof. em. dr. Baron de Barsy,
directeur scientifique
Bruxelles, 31 décembre 2009

Universiteiten met onderzoeksprogramma's die gesteund worden door de G.S.K.E.

Universités ayant des programmes de recherche subventionnés par la F.M.R.E.

Universities having research programs supported by the Q.E.M.F.



Onderzoeksprogramma's gefinancierd door de G.S.K.E. - Programma 2008-2010

Programmes de recherche subventionnés par la F.M.R.E. - Programme 2008-2010

Q.E.M.F. funded research projects - Program 2008-2010

K.U.Leuven

- **Prof. dr. Peter Carmeliet**
  Unraveling the role and therapeutic potential of Flt1 receptor ligands in amyotrophic lateral sclerosis (ALS).

- **Prof. dr. Peter Janssen**
  The presentation of three-dimensional shape in posterior parietal and premotor cortex of the rhesus monkey.

- **Prof. dr. Wim Vanduffel**
  Large-scale causal functional interactions between cortical areas: from anatomy to neuro-pharmacology.

- **Prof. dr. Rufin Vogels**
  Coding of biological motion in macaque monkeys: relating perception and neuronal selectivity.

UA

- **Prof. dr. Vincent Timmerman**
  Molecular genetics and biology of Charcot-Marie-Tooth neuropathies.

- **Prof. dr. Christine Van Broeckhoven**
  Progranulin in neurodegenerative dementia: genetic, functional and neuropathological characterization.

UCL

- **Prof. dr. André Goffinet**
  Genetic, molecular and cellular mechanisms of cortical development.

- **Dr. Emmanuel Hermans**
  Cellular crosstalks in amyotrophic lateral sclerosis: influence of neuroinflammation on astrocyte function and stem cell differentiation.

UGent

- **Prof. dr. Frans Van Roy**
  Functional analysis of novel adhesive and signaling proteins in development and tumorigenesis of neural tissues.

ULB

- **Prof. dr. Marc Parmentier**
  Characterization of G protein-coupled receptors involved in drug addiction and motor diseases.

- **Prof. dr. S.N. Schiffmann**
  Roles of specific neuronal populations in functions and disorders of basal ganglia: a transgenic and molecular approach.

- **Dr. Pierre Vanderhaeghen**
  Mechanisms of the development and evolution of the cerebral cortex.

ULg

- **Prof. dr. Pierre Maquet**
  Characterization of spontaneous brain activity in unconscious participants by multimodal functional neuroimaging.

- **Prof. dr. Gustave Moonen**
  Characterization of new cellular and molecular mechanism underlying migration of interneurons in the telencephalon.

UMH

- **Dr. Laurence Ris**
  Role of protein synthesis in late long-term potentiation (L-LTP).

VUB

- **Prof. dr. Yvette Michotte**
  Exploration of the memory enhancing effects of angiotensin IV and unravelling its mechanism of action.

Progress reports of the university research groups, supported by the Queen Elisabeth Medical Fondation in collaboration with the following professors and doctors (2009)

Progress report of the research group of

Prof dr. P. Carmeliet

Katholieke Universiteit Leuven (K.U.Leuven)

Applicant:

Carmeliet Peter (Main Applicant)

Applicant's Coordinator:

Lambrechts Diether (Co-Applicant)

Name of Institution: (main applicant only):

Vesalius Research Institute
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Unraveling the role and therapeutic potential of Flt1 receptor ligands in amyotrophic lateral sclerosis (ALS)

Introduction

During the first year of the proposal we investigated the neuroprotective role of VEGF-B in motoneuron degeneration and established that VEGF-B protects against mutant SOD1-mediated motor neuron degeneration (amyotrophic lateral sclerosis). These findings were published in the Journal of Neuroscience (Poesen et al., 2009). In the second year of the grant (for which we are here providing the report), we investigated whether VEGF-B is also neuroprotective in other models of neurodegeneration. In particular, we investigated whether VEGF-B has therapeutic effects in models of sensory neuropathies. We have been able to demonstrate that the neuroprotective activities of VEGF-B are not restricted to motor neurones but also extend to sensory neurons. The data are described in detail below.

Results

Expression of VEGF-B: For VEGF-B to be involved in the sensory nervous system innervating the footpads of the hindlimbs, it has to be expressed in the corresponding dorsal root ganglia (DRGs) descending from the lumbar spinal cord at level L4 to L5. We therefore performed immunohistochemistry to show clear expression of VEGF-B in the neurons of the DRGs (Figure 1A-C). Expression of VEGF-B was confirmed by RT-PCR on whole DRGs and isolated primary DRG neurons.

VEGF-B is Dispensable for Sensory Neuron Survival. Despite the expression of VEGF-B by adult DRG neurons, survival of cultured primary DRG neurons isolated from adult VEGF-B$^{-/-}$ mice was normal compared to WT mice after 1 and 7 days of culture. This indicates that the endogenous production of VEGF-B by sensory neurons was not critical for their survival (n=7-7; P=NS; Figure 1D). VEGF-B was also dispensable for sensory nervous system survival during the development of the hindlimbs, since staining with panaxonal marker PgP9.5 showed that the density of the sensory axons in the dermis from VEGF-B$^{-/-}$ footpads did not differ from that of WT mice (n=3-3; P=NS; Figure 1E-F). Nor did VEGF-B$^{-/-}$ mice exhibit signs of functional sensory abnormalities.

VEGF-B Deficiency does not Affect the Footpad Vasculature. Consistent with previous reports, mice lacking VEGF-B exhibited comparable vascular densities as shown after tail vein injection of a fluorescent dextran (n=5-5; P=NS) and whole-mount immunostaining for the vessel marker CD31 in the dermis of footpads (n=6-5; P=NS; Figure 1G-H). Overall, these data indicate that VEGF-B is dispensable for the development and function of the sensory nervous and vascular system, at least under healthy conditions.

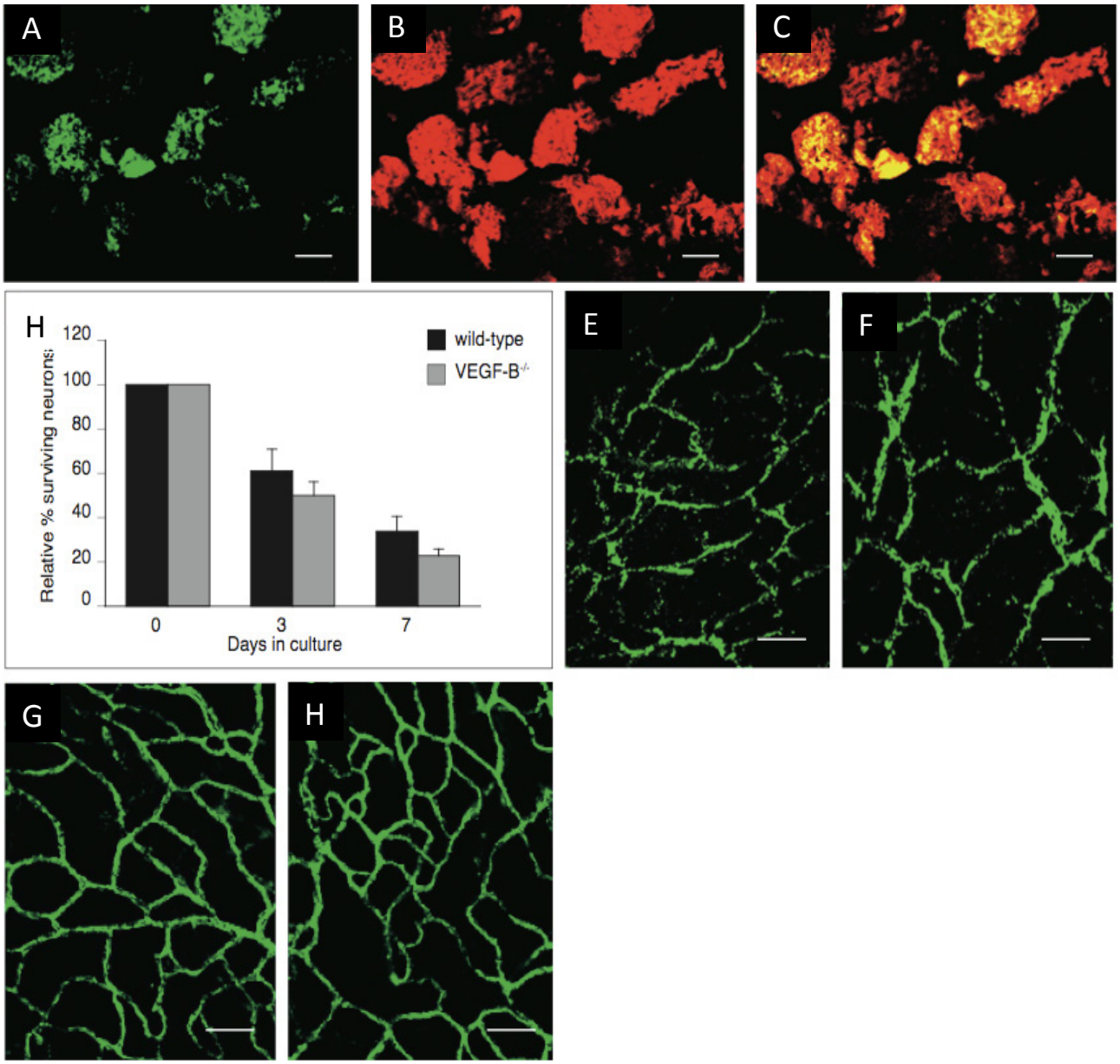

Figure 1: VEGF-B is dispensable for the sensory nervous system. (A-C) VEGF-B expression is detected on DRG neurons as revealed by double immunostaining on cryo-unfixed sections for endogenous VEGF-B (A) and the neuronal marker NeuN (B). (C): merged picture. **(D)** The number of surviving primary DRG neurons isolated from WT and VEGF-B$^{-/-}$ mice did not differ after 0, 3 and 7 days in culture (P=NS; n=7 preparations from n=3 mice per strain). **(E-F)** Whole-mount preparations of footpads from wild-type (E) and VEGF-B$^{-/-}$ (F) mice stained for the pan-axonal PgP9.5 marker reveals that there were no differences in the density of innervating sensory nerve axons (n=3-3; P=NS). **(G-H)** Staining for the endothelial CD31 marker reveals that there also were no differences in the vascular density of these footpads (n=5-5; P=NS). Scale bar (A-C): 25 µm. Scale bar (E-H) :50 µm.

VEGF-B$^{-/-}$ DRGs Display more Neuronal Stress in Vitro. Since it is possible that deficiency for VEGF-B increases neuronal stress without affecting survival or functionality, we quantified the expression of the activating transcription factor 3 (ATF3) as a measure of neuronal stress (1, 2). When primary DRG neurons isolated from VEGF-B$^{-/-}$ mice were cultured under standard conditions, the number of neurons immunopositive for ATF3 was increased compared to WT DRG cultures (n=7-7; P<0.001; Figure 2A). Furthermore, VEGF-B$^{-/-}$ DRGs were more sensitive to Taxol. Low concentrations of Taxol (i.e., from 10^{-9} M up to 10^{-7} M) were used, as they increase the number of ATF3-positive cells without affecting the survival of WT primary DRGs. When exposing DRGs from WT mice to increasing concentrations of Taxol, the number of ATF3 immunopositive cells gradually increased, reaching a maximal level after exposure to 10^{-7} M Taxol (Figure 2A). In contrast, ATF3 levels already reached their maximal level in

VEGF-B$^{-/-}$ DRGs exposed to 10^{-9}M Taxol. The addition of higher concentrations of Taxol to VEGF-B$^{-/-}$ cultures (i.e., 10^{-8} or 10^{-7} M) did, however, not affect the survival of these neurons. VEGF-B deficiency thus renders primary DRG neuronal cultures more susceptible to neuronal damage when cultivated under baseline and stressed conditions, suggesting a role for VEGF-B in neurosensorial protection.

Taxol Affects Vessels and Nerves in Taxol-induced Neuropathies. To further investigate the activities of VEGF-B under stressed *in vivo* conditions, we used a model of Taxol-induced sensory nerve degeneration in mice. In this model, cumulative injections of Taxol were given subplantarily into the left footpad during 4 consecutive days, leading to retrograde degeneration of intra-epidermal nerve fibers and the development of a sensory deficit, from which the mice gradually recovered once Taxol injections were arrested. To reliably and functionally assess this sensory deficit, pinprick scores, in which the footpad was punctured with a sharp pin and the withdrawal response of the hindlimb monitored, were used: a response to the pinprick was scored as 0, no response as 1.

Since VEGF-B can affect both the vascular and nervous system, we first investigated the effect of Taxol on vessels and axons in footpads. On the first day after the 4th Taxol injection, fewer vessels were perfused and less PgP9.5-positive axons were detectable in treated *versus* untreated footpads (n=3-3; $P<0.05$ and $P<0.01$ respectively). At the level of the DRGs innervating the footpads (i.e., L4 to L5), we failed to detect neuronal cell death after Taxol. A 10-fold increase in ATF3 (but not GFAP) expression level was, however, detectable in DRGs, indicating that the neurons in these DRGs suffered from increased neuronal stress, similar to primary DRG cultures challenged with low concentrations of Taxol.

Mice lacking VEGF-B are more Susceptible to Taxol-induced Neuropathy. We also challenged mice lacking VEGF-B with Taxol. VEGF-B$^{-/-}$ mice developed a more pronounced sensory deficit than their corresponding WT littermates (n=10-10; $P<0.05$; Figure 2B). Quantification of the PgP9.5-positive axons in the footpad also revealed that there were less sensory axons in VEGF-B$^{-/-}$ mice on the 1st day after the Taxol injections (n=4-8; $P<0.05$; Figure 2C-D). Perfused vessel area in the footpad did not differ between the genotypes (n=5-5; P=NS). Thus, deficiency for VEGF-B aggravated distal degeneration of sensory nerve endings without affecting the vasculature.

VEGF-B is Neuroprotective in DRG Cultures. Since our findings illustrate that DRG neurons lacking VEGF-B are more susceptible to Taxol-induced neuropathy, we analysed whether the aggravated phenotypes in these mice might be attributable to reduced neuroprotective activities of VEGF-B. To test this hypothesis, we added recombinant mouse VEGF-B to DRG cultures. We first investigated whether recombinant VEGF-B^{186} protein was capable of reducing neuronal stress. In combination with 10^{-8} M Taxol, VEGF-B^{186} reduced neuronal stress in a dose-responsive fashion ($r^2=0.99$ respectively after a sigmoidal fit; Figure 2E). Furthermore, VEGF-B^{186} was capable of protecting DRG neurons against Taxol-induced cell death. Primary DRG cultures were challenged with a high Taxol dose (10^{-6} M), which induced cell death of DRG neurons. After 6 days, 10^{-6} M Taxol caused increased cell death that was counteracted by addition of 50 ng VEGF-B^{186} (n=6 per condition; $P<0.05$; Figure 2F). All together, these data clearly illustrate that VEGF-B is a direct neuroprotective factor for DRG neurons *in vitro*.

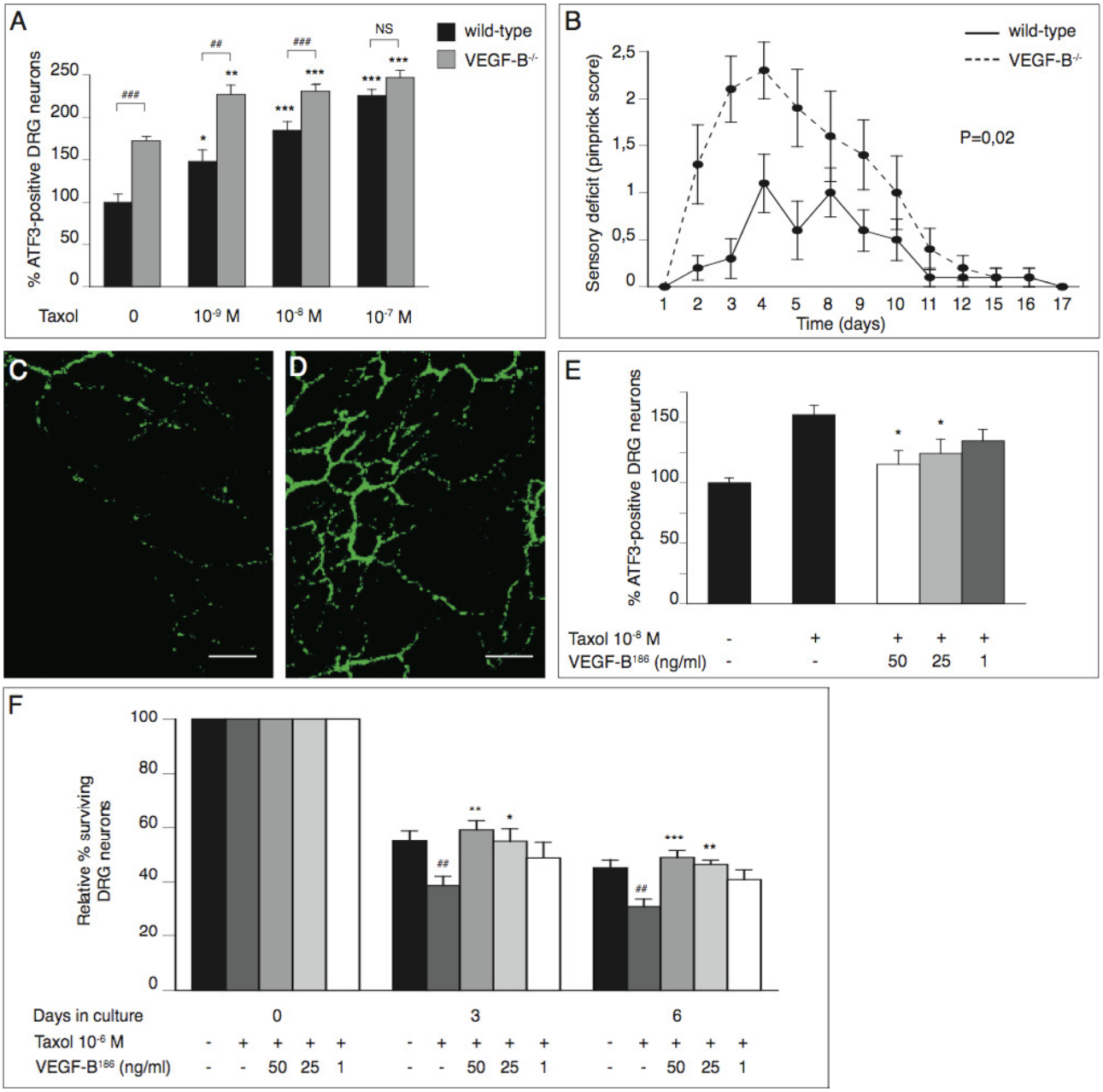

Figure 2: VEGF-B deficiency increases susceptibility to neuronal stress. (A) The percentage of ATF3-positive DRG neurons isolated from wild-type and VEGF-B$^{-/-}$ mice was 73% higher in DRG cultures lacking VEGF-B in baseline conditions. When exposed to different concentrations of Taxol (i.e., 10^{-9} M, 10^{-8} M and 10^{-7} M), wild-type cultures exhibited maximal ATF3 levels when exposed to 10^{-7} M Taxol. In contrast, VEGF-B$^{-/-}$ cultures already showed a maximal ATF3 response with 10^{-9} M Taxol. Asterisks denote p-values compared to untreated cultures: *: p<0.05, **: p<0.01 and ***: p<0.001; and # denote p-values compared to WT: #: p<0.05, ##: p<0.01 and ###: p<0.001 (n=6 preparations from n=3 mice per strain). **(B)** Mice lacking VEGF-B develop a more pronounced sensory deficit in a model of Taxol-induced neuropathy compared to wild-type mice (P=0.02). Averaged pinprick scores (mean ± SEM) are shown to visualize the sensory deficit in time (n=10 mice per genotype).**(C-D))** Quantification of the PgP9.5-positive axons in footpads from VEGF-B$^{-/-}$ mice (C) reveals that there were less axons on the 1st day after the Taxol injections compared to WT footpads (D) (P=0.023; n=4-8). **(E)** Addition of 3 different VEGF-B^{186} concentrations (1, 25 and 50 ng/mL VEGF-B^{186}) dose-dependently protects primary DRG neurons from neuronal stress induced by 10^{-8} M Taxol (n=6 preparations from n=3 rats per condition)**.** Asterisks denote p-values compared to untreated cultures: *: p<0.05, **: p<0.01. **(F)** VEGF-B^{186} also protects DRG cultures from cell death induced by 10^{-6} M Taxol. Asterisks denote p-values compared to 10^{-6} M Taxol cultures (n=6 preparations from n=3 rats per condition). Asterisks denote p-values compared to untreated cultures: *: p<0.05, **: p<0.01 and ***: p<0.001, and # denote p-values compared to cultures not receiving Taxol: #: p<0.05, ##: p<0.01 and ###: p<0.001. Scale bars (C-D)=50 µm.

Expression of Flt1 in the Sensory Nervous System. For VEGF-B to exert these effects through its selective receptor Flt1, this receptor should be expressed on DRG neurons. Double-immunohistochemistry showed that Flt1 was expressed in DRG neurons (Figure 3A). This was confirmed by RT-PCR on whole

DRGs and isolated primary DRG neurons. Weak expression of Flt1 was also seen in blood vessels. Thus, the molecular players necessary for VEGF-B to exert its direct neuroprotective effects are present in the sensory nervous system.

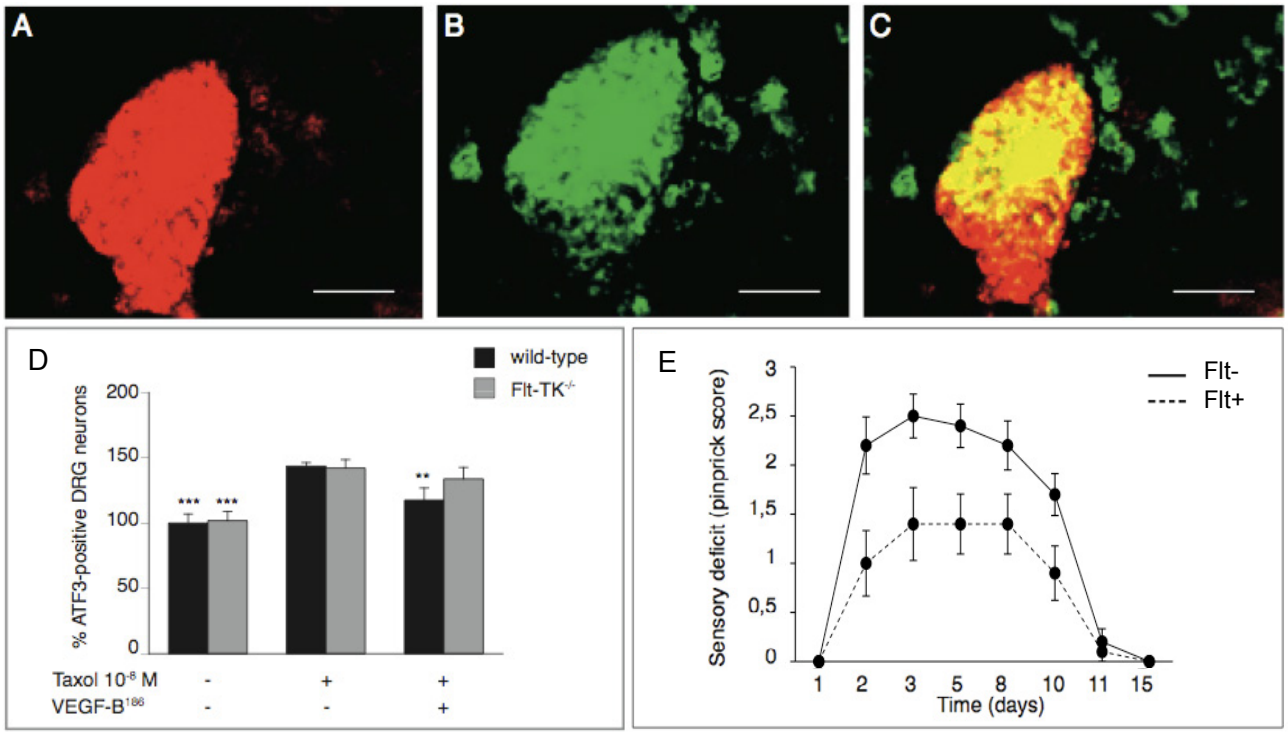

Figure 3: Flt1 is a functional VEGF-B receptor in the nervous system. (A-C) Flt1 expression is detected on DRG neurons as revealed by double immunostaining on cryo-unfixed sections for endogenous Flt1 (A) and the neuronal marker NeuN (B). (C): merged picture. **(D)** The neuroprotective effects of VEGF-B^{186} on DRG neurons depend on the tyrosine-kinase activities of Flt1: recombinant VEGF-B^{186} (50 ng/mL) is capable of reducing ATF3 immunoreactivity in primary DRG neurons from WT mice (P<0.01), but not in primary DRG cultures isolated from Flt-TK$^{-/-}$ mice (P=NS; n=12 preparations from n=3 mice per genotype). Asterisks denote p-values compared to untreated 10^{-8} M Taxol cultures: *: p<0.05, **: p<0.01 and ***: p<0.001. **(E)** Mice overexpressing Flt1 in postnatal neurons develop a less pronounced sensory deficit in a model of Taxol-induced neuropathy compared to Flt1^{-} mice (P<0.005). Averaged pinprick scores (mean ± SEM) are shown to visualize the sensory deficit in time (n=9-10 mice). Scale bars (A-C)=25 µm.

VEGF-B exerts Neuroprotective Effects through Flt1. To investigate whether the neuroprotective effect of VEGF-B^{186} was indeed mediated through Flt1, we used mice expressing a tyrosine-kinase dead Flt1 (Flt-TK$^{-/-}$ mice). In contrast to wild-type cultures, VEGF-B^{186} failed to protect Flt-TK$^{-/-}$ DRG cultures from Taxol-induced neuronal stress (n=6 per condition; P=NS; Figure 4B). As these *in vitro* studies identified Flt1 as the receptor responsible for neuroprotective effects of VEGF-B, we challenged Flt-TK$^{-/-}$ mice subplantarily with a low Taxol dose. WT mice developed a milder sensory deficit compared to Flt-TK$^{-/-}$ mice (n=12-9; P<0.05). As described for VEGF-B$^{186/+}$ mice, mice overexpressing Flt1 in postnatal neurons (Flt1^{+} mice) were generated to assess the beneficial effect of neuronal Flt1 overexpression on sensory neuropathy. Flt1^{+} mice displayed 20% more Flt1 expression in whole DRGs (n=10-8; P<0.05) compared to Flt1^{-} mice. This neuronal overexpression of Flt1 protected mice against Taxol-induced neuropathy induced in the footpad (n=9-10; P<0.005; Figure 3E). Thus, overexpression of Flt1 in sensory neurons also protects against Taxol-induced neuropathy, whereas TK dead Flt1 receptor mice develop more severe Overall, these findings demonstrate that Flt1 transmits the neuroprotective signals from VEGF-B on sensory neurons.

Overexpression or Delivery of VEGF-B^{186} Reduces Taxol-induced Axonopathy. To explore whether VEGF-B was also capable of protecting against Taxol-induced neuropathies *in vivo*, we generated

a transgenic mouse specifically overexpressing VEGF-B^{186} in neurons (Figure 4A). First, knock-in mice were generated by inserting a floxed STOP sequence upstream of the VEGF-B^{186} cDNA into the ROSA26 locus of embryonic stem cells (3). Mice expressing this construct (i.e., $stop^{lox/lox}$VEGF-B^{186}) were intrecrossed with mice expressing the Cre recombinase postnatally under control of the neuronal Thy1.2 promoter (i.e., Thy1.2:Cre mice). Analysis of these VEGF-$B^{186/+}$ mice showed a normal density of sensory axons (n=7-7; P=NS) and a normal vasculature in the footpad (n=5-5; P=NS), thereby confirming our previous findings that VEGF-B is dispensable for normal sensory nerve function.

To subsequently assess the neuroprotective role of VEGF-B^{186} overexpression, Taxol was administered into the footpads of VEGF-$B^{186/+}$ mice and VEGF-$B^{186/-}$ littermates. Neuronal overexpression of VEGF-B^{186} significantly protected against the Taxol-induced neuropathy ($P<0.0005$; n=10-12; Figure 4B). This thus suggests that prophylactic administration of VEGF-B^{186} can prevent Taxol-induced neuronal damage. In a last experiment, we assessed whether subplantar injection of recombinant VEGF-B^{186} protein 1 hour prior to every Taxol injection, could protect mice against Taxol-induced neuropathy. VEGF-B^{186} had a clear inhibitory effect on the development of the neuropathy (n=7-7; $P<0.05$; Figure 4C). To assess whether the therapeutic effect of recombinant VEGF-B^{186} was due to angiogenic or direct neuroprotective effects, we quantified the degree of vessel perfusion: after the 4^{th} Taxol injection, a similar amount of vessels was perfused in VEGF-B^{186} and PBS-treated mice (n=3-3; P=NS). Similar concentrations of the key angiogenic factor VEGF induced a potent vessel-promoting effect in these footpads.

Altogether, these findings indicate that VEGF-B is a direct neuroprotective factor for sensory neurons, independent of angiogenesis. In addition, VEGF-B offers therapeutic perspectives for the prevention and treatment of neurotoxic disorders affecting sensory neurons.

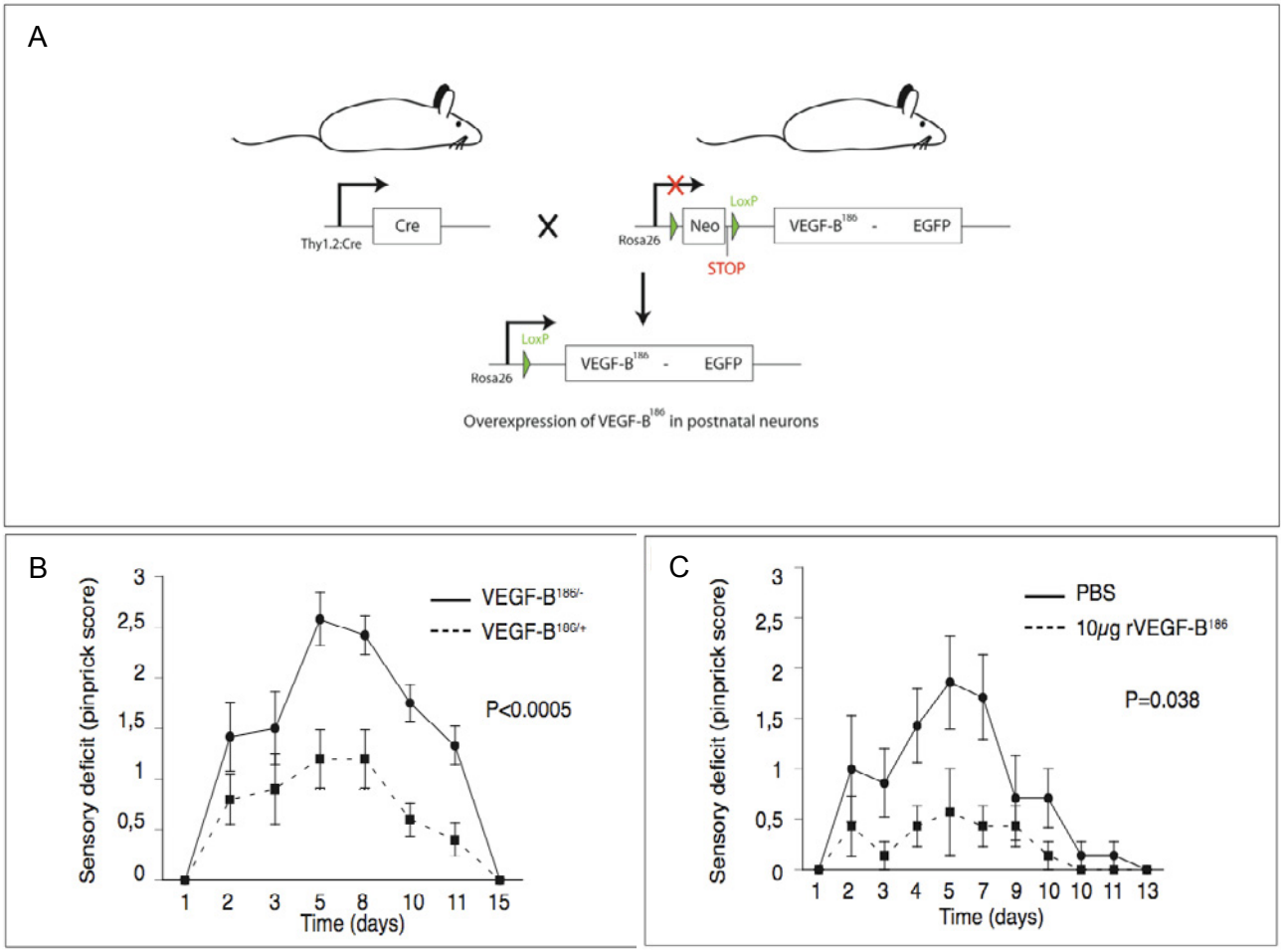

Figure 4: Therapeutic potential of VEGF-B^{186} for Taxol-induced neuropathies. (A) Strategy for the generation of a transgenic mouse line, in which VEGF-B^{186} is selectively overexpressed in postnatal neurons. The VEGF-B^{186} cDNA is cloned downstream of a floxed STOP sequence and upstream of an IRES sequence followed by an enhanced green fluorescent protein (eGFP) marker. Transgenic mice expressing this construct under control of the endogenous ROSA promoter are subsequently generated (stop$^{lox/lox}$VEGF-B^{186} mice). In the absence of Cre, ROSA promoter activity is blocked by an in-frame STOP sequence resulting in no overexpression of VEGF-B (VEGF-B$^{186/-}$ mice). In the presence of the Cre protein, which is expressed in postnatal neurons under control of the Thy1.2 promoter, the STOP sequence is excised resulting in increased expression of VEGF-B^{186} (VEGF-B$^{186/+}$ mice). **(B)** Mice overexpressing VEGF-B^{186} (VEGF-B$^{186/+}$ mice) develop a less pronounced sensory deficit in a model of Taxol-induced neuropathy compared to VEGF-B$^{186/-}$ mice ($P<0.0005$). Averaged pinprick scores (mean ± SEM) are shown to visualize the sensory deficit in time (n=10-12 mice). **(C)** Mice receiving 10 µg recombinant VEGF-B^{186} develop a less pronounced sensory deficit in a model of Taxol-induced neuropathy compared to PBS-injected control mice ($P<0.05$). Averaged pinprick scores (mean ± SEM) are shown to visualize the sensory deficit in time (n=7-7 mice).

Summary

Recent studies have shown a *in vitro* and *in vivo* neuroprotective effect for VEGF-B in the central nervous system (4-7). We provide unprecedented evidence that VEGF-B also exerts neuroprotective effects in the sensory nervous system. The effects of VEGF-B were restricted to pathological conditions, indicating that VEGF-B is not required for the survival of sensory neurons in healthy conditions. However, DRG neurons isolated from adult VEGF-B$^{-/-}$ mice exhibited increased neuronal stress in normal conditions and were also more sensitive to Taxol-induced neuronal stress. Furthermore, in an *in vivo* model of Taxol-induced sensory nerve degeneration, VEGF-B deficient mice displayed a more severe sensory deficit. When added to primary DRG cultures, VEGF-B protein reduced neuronal stress and increased the survival of primary DRG neurons. Altogether, these data show that VEGF-B mediates sensory nerve function by reducing Taxol-induced neuronal stress and cell death.

Intriguingly, VEGF-B did not seem to act on the vasculature. Indeed, the number of perfused vessels in VEGF-$B^{-/-}$ mice challenged with Taxol was similar to that of Taxol treated WT mice. Local delivery of recombinant VEGF-B^{186} was also protective against Taxol without eliciting any obvious effect on the vasculature. Rather than acting as an angiogenic and neuroprotective factor, like VEGF, the effects of VEGF-B in the sensory nervous system are thus only neuroprotective in nature. Another finding from our study is that these effects of VEGF-B were mediated through its Flt1 receptor. Indeed, primary DRG neurons isolated from mice lacking a tyrosine-kinase Flt1 receptor failed to respond to recombinant VEGF-B^{186}. In summary, our data indicate that VEGF-B counteracts Taxol-induced neuropathies and stimulate further research to investigate the therapeutic utility of VEGF in the (sensory) nervous system.

Publications in which funding by GSKE was mentioned:

- Novel role for vascular endothelial growth factor (VEGF) receptor-1 and its ligand VEGF-B in motor neuron degeneration. Poesen K, Lambrechts D, Van Damme P, Dhondt J, Bender F, Frank N, Bogaert E, Claes B, Heylen L, Verheyen A, Raes K, Tjwa M, Eriksson U, Shibuya M, Nuydens R, Van Den Bosch L, Meert T, D'Hooge R, Sendtner M, Robberecht W, Carmeliet P. J Neurosci. 2008 Oct 15;28(42):10451-9.
- Meta-analysis of VEGF variations in ALS: increased susceptibility in male carriers of the -2578AA genotype. Lambrechts D, Poesen K, Fernandez-Santiago R, Al-Chalabi A, Del Bo R, Van Vught PW, Khan S, Marklund S, Brockington A, Van Marion I, Anneser J, Shaw C, Ludolph A, Leigh N, Comi G, Gasser T, Shaw PJ, Morrison K, Andersen P, Van den Berg LH, Thijs V, Siddique T, Robberecht W, Carmeliet P. J Med Genet. 2008 Jul 17. [Epub ahead of print]
- A third manuscript reporting on the neuroprotective effects of VEGF-B in sensory neuropathies is currently in preparation

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Genetic, molecular and cellular mechanisms of cortical development

Background

In 2009, work supported by the FMRE in our laboratory has been mostly focused on the role of the seven pass cadherins Celsr1, Celsr2 and Celsr3. We have also completed a study of the action of oncogene p73 during brain development and in tumor progression. We also worked on the production and preliminary characterization of mice with invalidation of Lrrn1-3, three homologous neuronal receptors containing leucine-rich repeats.

1. Role of the seven pass cadherins Celsr1-3 in cortical development

Celsr1-3 are a family of three mammalian gene orthologs of Flamingo (Tissir et al., 2002a; 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. Previous studies in the laboratory have addressed the role of Celsr3 (Tissir et al., 2005; Zhou et al., 2008). This has been extended with studies of the maturation of “cortex isolé”in vivo. Furthermore, in 2009, we have studied ependymal development and the physiology of hydrocephalus in Celsr2 and Celsr3 mutants, development of facial branchiomotor neurons in all Celsr1-3 mutants, and skin development in Celsr1 mutant mice.

“Neocortex isolé” *in vivo*.

We previously showed that Celsr3|Dlx mice have no internal capsule. Yet, animals are able to survice up to P20, when cortical maturation is complete, in the absence of corticothalamic, thalamocortical and subcortical connections. We have analyzed that malformation in detail and shown that the protomap forms normally and that cortical lamination also proceeds normally. During maturation, absence of connections results in neuronal loss, predominantly in deep layers 5 and 6. Surviving neurons are atrophic but with consistent morphological features. In collaboration with the teams of S. Schiffmann (ULB) and G. Cheron (ULB), we showed that neurons in cortex isolé are hypo-excitable in vitro, but fire almost normally in vivo. Furthermore, some cortical oscillations occur in this cortex. The manuscript describing these observations is submitted and under review (Zhou et al.).

Ependymal and hydrocephalus in Celsr2 and double Celsr2+3 mutants.

Constitutive Celsr2 mutant allele survive and most of them are fertile. They develop progressive hydrocephalus, which is exacerbated in animals that are also mutated for Celsr3 in cortex (Emx1-Cre). We attribute this to defective planar cell polarity in the ependyma and have studied cilia and ependymal markers in Celsr2, Celsr2+3 and control mice with scanning EM as well as transmission EM, and in vitro systems to analyze cilia beats. That study has been submitted for publication and a revised manuscript will be proposed in early 2010.

Phenotype of Celsr1 mice.

We have produced a Celsr1 conditional mutant, from which we derived a constitutive mutant using crosses with PGK-Cre mice. Heterozygotes are normal, and homozygotes have a variable phenotype,

probably strongly influenced by genetic background. Some animals die during embryonic development, from defective neural tube closure. Others survive to early postnatal period, and some to adult stages. Adult Celsr1 mutants have an intriguing hair whorl phenotype on head, feet and body. Interestingly, this is similar to the skin phenotype observed in homozygous Fzd6 mutants, indicating that, like Celsr3 and Fzd3, Celsr1 and Fzd6 act in a common genetic pathway. The study of hairs in Celsr1 mutant has been published (Ravni et al., 2009).

Migration of facial branchiomotor neurons

Following a study implicating Celsr genes in facial motor migration in zebrafish, we have studied in detail the migration of facial motoneurons in our three Celsr mutant mice. In wildtype animals, facial motoneurons are generated in rhombomere 4 and migrate through rhombomere 5 to reach rh6 where they form the facial nucleus. This migration is defective in Celsr1 mutants, where the direction of migration is drastically perturbed, as well as in Celsr2 mutants, where the trajectory of caudal migration is disturbed. Migration is normal in Celsr3, but there is neuronal death in double Celsr2+3 mutants. By studying double Celsr1+2 mutants, we could show that Celsr2 is epistatic to Celsr1. Studies of Celsr1|Isl1 mice with conditional inactivation of Celsr1 using crosses with Islet1-Cre mice showed that the action of Celsr1 is not cell autonomous. This study was made in collaboration with the laboratory of Anand Chandrasekhar (U. Missouri, USA) and is still pursued by Yibo Qu in our laboratory. A first manuscript is currently submitted.

2. Role of oncogene DNp73 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 and fully validated 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.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 have also shown that DNp73 is heavily expressed in the accessory olfactory bulb and the organ of Jacobson, as well as in the embryonic thalamic eminence. By crossing our p73 allele with ROSA-DTA mice, we have genetically ablated p73 expressing cells and this confirmed expression in subsets of cells. We also showed that DeltaNp73 is a marker for the thalamic eminence, a neurogenic region that contributes neurons to basal telencephalon and was not widely known. Observations on the role of DeltaNp73 in brain development have been published (Tissir et al., 2009). We have also tested whether DNp73 acts as an oncogene in vivo by injecting wildtype and DNp73 -/- mice with methylcholanthrene. That study allowed us to show that DeltaNp73 influences tumor development as predicted, but the effect is modest and limited to female mice. These data have been submitted (Ravni et al., Cell Cycle, accepted pending minor revisions). We have also undertaken to study brains of DeltaNp73 mutant mice aged more than one year, to test whether neurofibrillary tangles accumulate in such mutants. This is done in collaboration with Prof. JP Brion (ULB).

3. Lrrn1-3

Lrrn1-3 are a family of three closely related genes that encode membrane receptors with leucine-rich repeats that are heavily and preferentially expressed in the brain, whence the acronym that stands

for “Leucine-rich repeat receptor neuronal” (Taguchi et al., 1996; Almeida et al., 1998; Hamano et al., 2004; Garcia-Calero et al., 2006; Andreae et al., 2007). This family attracted our attention after a role in development was demonstrated for two ortholog genes in Drosophila, named tartan and capricious (Milan et al., 2001; Krause et al., 2006; Mao et al., 2008). Last year, we reported production of conditional and constitutive mutants for Lrrn1 and Lrrn3. In 2009, we also produced double Lrrn1+3 constitutive mutants. Both Lrrn1, Lrrn3 as well as Lrrn1+3 mutant animals are viable and fertile. A preliminary examination of brains on paraffin, Nissl stained sections did not disclose any obvious pathology, indicating that the three genes have redundant actions, which is not unexpected given the very important similarity in sequence and genomic organization and the large overlap of expression patterns. We have successfully targeted ES cells with a conditional Lrrn2 construct, and performed several sequences of microinjection in blastocysts. We have obtained several chimeras, none of which was able to transmit the mutant allele in the germline thus far. Further injections are carried out and will be planned in the beginning of 2010. Unfortunately, these unforseen difficulties in obtaining the Lrrn2 mutant have resulted in a significant delay in this project, which is not expected to lead to a publication before 2011 or even 2012.

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Cellular crosstalks in amyotrophic lateral sclerosis : influence of neuroinflammation on astrocyte function and stem cell differentiation

The importance of glial cells in the physiology of the central nervous system has been largely demonstrated. During the last decades, glial cells have also received considerable attention with respect to their putative role in the development and/or the progression of neurological disorders. Indeed, the innate immune protection of the CNS ensured by microglia might be compromised in pathological conditions, leading to a neurotoxic phenotype of activated cells with a robust inflammatory profile. Microglia activation is accompanied by the release of a variety of pro-inflammatory cytokines and free radicals which actively participate in the degenerative process. Besides, excitotoxic neuronal damages frequently result from impaired handling of extracellular glutamate by astrocytes participating in gliosis. As microglia is recognised as a potent inducer of astrocyte activation, pro-inflammatory mediators are expected to modulate the glutamatergic transmission by regulating glutamate receptors and transporters. Three major excitatory amino acid transporter (EAAT) subtypes termed GLAST (glutamate aspartate transporter), GLT-1 (glutamate transporter-1) and EAAC1 (excitatory amino acid carrier-1) have been cloned in the rat forebrain and are the rodent homologues of the human EAAT1-3, respectively. Even though GLAST is the predominant glutamate transporter during brain development, GLT-1 is the most abundant subtype in mature organisms and is responsible for up to 90% of the total glutamate clearance in adult tissues. Regulation of GLT-1 has been extensively investigated, as it exhibits the highest affinity for the substrate and thereby efficiently contributes to protect neurons against excitotoxic insults. Hence, alterations in its expression or activity have been reported in several pathological conditions such as amyotrophic lateral sclerosis, multiple sclerosis or ischemia.

The research projects conducted in the laboratory concern the study of the (dys-) regulation of the glutamate transporter GLT-1 in glial cells with a special focus on the role of inflammation and the importance of this regulation in the progression of selected nervous disorders (amyotrophic lateral sclerosis and neuropathic pain) :

- regulation of GLT-1 isoforms in astrocytes derived from a model of amyotrophic lateral sclerosis
- the role of caspases 3 in the regulation of glutamate uptake in astrocytes specifically derived from the white matter of rodents developing amyotrophic lateral sclerosis.
- the importance of inflammation and glutamate transmission on the development of neuropathic pain
- the importance of impaired glutamate uptake in the progression of brain tumours

Distinct expression and regulation of the glutamate transporter isoforms GLT-1a and GLT-1b in cultured astrocytes from a rat model of amyotrophic lateral sclerosis (hSOD1G93A)

Partially published in Goursaud S, Maloteaux JM, Hermans E. Distinct expression and regulation of the glutamate transporter isoforms GLT-1a and GLT-1b in cultured astrocytes from a rat model of amyotrophic lateral sclerosis (hSOD1G93A). Neurochem Int. 2009 Jul-Aug;55(1-3):28-34.

The existence of distinct isoforms of the GLT-1 has been demonstrated for more than 7 years. However, the study of the major isoforms (GLT-1a and GLT-1b) in nervous disorders has received little attention. Impaired glutamate uptake associated with accumulation of extracellular glutamate is a well documented feature of amyotrophic lateral sclerosis (ALS) and related excitotoxicity is frequently proposed to participate in the progression of the disease. We have characterised the expression and activity of the glutamate transporter GLT-1 in cultured cortical astrocytes derived from a transgenic rat strain expressing an ALS-related mutated form of human superoxide dismutase 1 (hSOD1G93A). Measurements of D-[3H]-aspartate uptake velocity in the presence of selective glutamate transporter blockers demonstrated that astrocytes from the transgenic rats showed an impaired GLT-1 activity as compared to cells from wild-type animals. In addition, the density of GLT-1a mRNA in cells from hSOD1G93A animals appeared nearly 2-fold lower while the density of GLT-1b mRNA was nearly 2-fold higher. This may well suggest that the poorly characterized GLT-1b isoform could play a major role in adaptive processes that develop during pathologic indults.

Therefore, we have also study the possibility to pharmacologically modulate the expression of the two GLT-1 isoforms. We observed that exposing the astrocytes from hSOD1G93A rats to the neuroprotective transmitter Peptide Histidine Isoleucine (PHI) for 24 h caused a 4.5-fold increase in the GLT-1b mRNA level without influencing the expression of the other key isoform GLT-1a. This selective upregulation of GLT-1b by the neuropeptide was correlated with a significant increase in D-[3H]-aspartate uptake activity. The possibility to specifically regulate a single isoform of the high-affinity transporter GLT-1 is an unprecedented observation which sheds light on new perspectives for the pharmacological manipulation of glutamate transmission in diseases such as ALS.

Reduction of caspase-3 activity upregulates the glutamate transporter GLT-1 in cultured white matter astrocytes from a rat model of amyotrophic lateral sclerosis (hSOD1G93A)

In preparation (Goursaud and Hermans)

Amyotrophic lateral sclerosis (ALS) is typically characterized by a dramatic loss of lower motor neurons in spinal cord and brainstem. Nevertheless, upper motor neuron dysfunction is also reported in ALS and frequently related to a deficit of transcallosal connections associated to a reduced volume of the corpus callosum. Besides, impairment of the astroglial glutamate transporter GLT-1 associated with accumulation of extracellular glutamate is demonstrated in ALS and related excitotoxicity is likely to participate in the progression of the disease. At the molecular level, the caspase-3-mediated cleavage of GLT-1 leading to a selective and functional inhibition of the transporter was evidenced in spinal cord homogenates of a transgenic mice model of ALS.

We have characterised the expression and activity of GLT-1 and caspase-3 in cultured callosal astrocytes isolated from a transgenic rat strain expressing an ALS-related mutated form of human superoxide dismutase 1 (hSOD1G93A). Quantitative RT-PCR and Western-blotting studies revealed that the expression of GLT-1 was higher in the cells prepared from the transgenic animals in comparison to the wild-type rats. However, specific measurements of D-[3H]-aspartate uptake velocity failed to evidence differences in the activity of this transporter. Nevertheless, measures of uptake in the presence of a caspase-3 inhibitor suggested that a reduced activity of this apoptotic enzyme, which is highly detected in callosal astrocytes from hSOD1G93A rats, could exclusively upregulate the GLT-1 activity in cells from

transgenic animals. Together, these findings reinforce the hypothesis of an involvement of caspase-3-mediated impairment of glutamate uptake in the pathogenesis of ALS.

The importance of inflammation and glutamate transmission on the development of neuropathic pain

In preparation (Berger and Hermans)

Glutamate is a key excitatory neurotransmitter involved in the processing of pain stimuli in the spinal cord. This neuronal signalling is regulated by astrocytes which express a variety of functional metabotropic glutamate receptors and glutamate transporters. Changes in the expression of these targets have been proposed to participate in the onset or maintenance of neuropathic pain. Following nerve injury, these changes in astroglial phenotype are thought to result from the influence of activated microglia which release a variety of chemical inducers. The aim of this part of our studies is to investigate whether factors released by activated microglia induce regulation of glutamatergic targets in astrocytes. Indeed, this hypothesis is supported by our previous in vitro studies indicating that inflammatory signals from activated microglia influence astroglial glutamate phenotype. Microglial activation was modelled in vitro by exposing primary cultures of microglial cells to lipopolysaccharide (LPS). After 48h, real-time quantitative PCR (q-PCR) of selected markers were performed to validate the activated status of microglia. In parallel, the conditioned media from these cells were transferred to primary cultures of astrocytes. On these cultures, the expression of the receptors (mGluR3, mGluR5) and transporters (GLAST, GLT1) was examined after 72h by q-PCR. Robust microglial activation by LPS was evidenced, as shown by concentration-dependent increases in transcripts of the pro-inflammatory enzymes iNOS and COX-2, and the cytokines TNF, IL-1b, or IL-6. The conditioned medium collected from these activated microglia was found to induce an opposite regulation of the glutamate receptors on astrocytes, as mGluR3 was upregulated while mGluR5 was downregulated. Moreover, GLT1 was upregulated while GLAST was unaffected. Our data suggest that the genetic expression of key proteins involved in glutamate handling by astroglia can be altered by factors released from activated microglia. Our next objective is to understand the mechanisms supporting these regulations and to elucidate the functional relevance of these findings to neuropathic pain. In this goal, we induce neuropathic pain in rodents through partial sciatic nerve ligation. Our experiments conducted in a model of inflammatory prone animals indicate that enhanced inflammation is correlated with exacerbated pain behaviour. Current works aim at examining the altered expression of glutamate transporters and receptors in these experimental conditions.

Enhanced expression of the high affinity glutamate transporter GLT-1 in C6 glioma cells delays tumour progression in rat

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Impaired functioning of glutamate transporters results in elevated extracellular glutamate concentration, a common feature of several nervous disorders, including motor neuron disease and Alzheimer's disease Indeed, excessive glutamatergic stimulation of neurons causes excitotoxicity which is physiologically

prevented by an active clearance process ensured by astroglial cells. Such implication of glutamate is also documented in other neurological disorders and microdialysis studies have evidenced elevated levels of extracellular glutamate in and around glial tumours in both patients and experimental animal models of glioma. Likely contributing to this excess of glutamate, reduced expression of glial glutamate transporters is a common feature of several human glioblastoma specimens and glioma cell lines. Notably, an inverse relationship between the expression of the most abundant glutamate transporter, the excitatory amino acid transporter 2 (EAAT2), and the tumour grade was recently confirmed.

In light of these observations, we generated C6 glioma cells in which expression of the glutamate transporter 1 (GLT-1, the rodent equivalent of EAAT2) could be manipulated in vivo, during tumour progression. Using this cellular model, we previously validated that enhancing glutamate transport activity of GLT-1 in C6 cells significantly reduced extracellular glutamate concentrations and significantly decreased cell proliferation in vitro. These data suggested that glutamate released by the XC- antiporter is rapidly cleared from the medium through efficient reuptake by GLT-1, decreasing glutamate-induced proliferation in C6 glioma cells. These cells were grafted in the striatum of Wistar rats and doxycycline was administered after validation of tumour development by magnetic resonance imaging. After validating the presence of a tumour, the expression of the transporter was induced and both tumour progression and animal survival were characterised. Both GLT-1 expression examined by immunohistochemistry and glutamate transport activity measured on synaptosomes appeared robustly increased in samples from doxycycline-treated animals. Moreover, these rats showed extended survival times as compared to vehicle-treated animals, an effect that was consistent with volumetric data revealing delayed tumour growth. These observations indirectly support the hypothesis that an intrinsic deficiency in glutamate transporter expression commonly observed in glioma cells is a key pathogenic feature of these glial tumours. This adds these malignant gliomas to the list of neurological disorders in which an excess of extracellular glutamate contributes to the progression of the disease. Moreover, these data also highlight the relevance of developing pharmacological approaches aiming at ensuring a better control of extracellular glutamate concentrations to reduce the invasive behaviour of glioma.

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The representation of three-dimensional shape in posterior parietal and premotor cortex of the rhesus monkey

We investigated to what extent AIP neurons and inferotemporal (IT) neurons functionally interact during the discrimination of disparity-defined 3D shapes. We observed strong synchronization in the beta band emerging 300 ms after stimulus onset. The degree of synchronization depended on the stimulus strength (the percentage of dots defining the surface). In the same study, we determined to what extent the neural activity in each of these areas correlates with the behavioral choice of the animal (choice probabilities), and assessed the effect of bilateral reversible inactivation of area AIP on 3D-shape discrimination. The choice probability rose early and during decision formation in inferotemporal cortex, but much later and after decision formation in area AIP. Hence the activity in IT, but not in AIP, correlates with behavioral choice during perceptual decisions about disparity-defined 3D-shapes. As a direct test of the role of AIP in the discrimination of 3D shape, we reversibly inactivated AIP in both hemispheres during 3D-shape discrimination. There was no effect on the behavioral performance, confirming the analysis of the choice probability data, but we did observe a marked deficit in grasping. These results indicate a role for AIP in the planning of grasping action but not in 3D-shape perception. They also demonstrate that synchronization between two brain areas is not always relevant for the task at hand. A first paper has been submitted to Current Biology.

Furthermore we investigated the coding of 2D- and 3D shape in parietal area AIP. We recorded single-unit activity in two passively fixating rhesus monkeys. The stimuli were images of real-world objects (both living and man-made objects) taken from two slightly different viewpoints. The two images of each object were presented alternately on a display to create the pattern of binocular disparity as present in the real object (=congruent disparity condition). To create the opposite pattern of disparities we exchanged the images between the eyes (= incongruent disparity condition). Control stimuli consisted of monocular presentations of the images, and binocular presentations of either the left-eye or the right-eye image. About half of the neurons tested (36/70, 51%) showed significant response differences between the congruent and the incongruent disparity condition that could not be explained by the monocular responses. A second test consisted of 2D presentations of either the original image (containing all texture and shading information), a silhouette stimulus which was entirely white, a contour stimulus consisting of the outer boundary, and a line-drawing stimulus. All neurons (N=37) were tested with an effective stimulus (the preferred shape) and an ineffective stimulus (the nonpreferred shape). The large majority of the neurons (31/37, 83%) showed selectivity for at least one of the 2D presentations. Surprisingly, most of these neurons (27/31, 87%) were selective for at least one of the 2D contour stimuli. These results demonstrate that AIP neurons do not require the presence of binocular disparity, and that for the majority of AIP neurons 2D contours are sufficient to evoke selective responses. Recordings in the second monkey are ongoing.

In a third study we investigated the coding of disparity-defined 3D shape in premotor area F5 of two rhesus monkeys. In total, 135 out of 230 responsive F5 neurons (59%) showed significant response differences between concave and convex surfaces that could not be accounted for by the monocular responses. The response latency of these F5 neurons was 70 ms, and the selectivity appeared already at 80 ms after stimulus onset. The large majority of the neurons tested (95/115, 83%) preserved their 3D-shape preference across positions-in-depth, hence showed higher-order disparity selectivity. We tested 77 higher-order neurons with curved surfaces containing varying disparity amplitudes (ranging from 0.03 to 1.3 deg). In monkey 1, the average tuning function showed a marked drop at the transition between concave and convex depth profiles, indicative of categorical coding of 3D shape similar to inferotemporal cortex. In monkey 2 the average tuning function was more monotonic. The recordings in the two animals are finished, and we are preparing the inactivation of area AIP during the recording of neural activity in F5.

Our previous studies strongly suggested that the shape coding in area AIP is not related to the perception of objects, but rather to the planning of grasping movements towards objects. We want to investigate the functional interactions between AIP and F5, and to assess the effect of reversible inactivation of area F5 on AIP. Our hypothesis is that motor dominant neurons in area AIP receive their information as a corrolary discharge from F5 neurons. Therefore we started training two rhesus monkeys in a delayed visually-guided grasping task. Six different objects can be presented on a carrousel, and the animal has to reach and grasp the object after the object is illuminated and a go-signal (an auditory tone) is given. The position of the hand is recorded using a Certus Optotrak system (NDI systems). One animal is trained in the task and the recordings will start in a few weeks.

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Characterization of spontaneous brain activity in unconscious participants by multimodal functional neuroimaging.

Regulation of sleep and wakefulness

Influence of the chronotype on daytime performance and its neural correlates

Throughout the day, cognitive performance is under the combined influence of circadian processes and homeostatic sleep pressure. Some people perform best in the morning whereas others are more alert in the evening. These chronotypes provide a unique way to study the effects of sleep/wake regulation on the cerebral mechanisms supporting cognition. Using functional magnetic resonance imaging in extreme chronotypes we found that maintaining attention in the evening was associated with higher activity in evening than morning chronotypes in a region of the locus coeruleus and in a suprachiasmatic area (SCA) including the circadian master clock. Activity in the SCA decreased with increasing homeostatic sleep pressure. This result shows the direct influence of the homeostatic and circadian interaction on the neural activity underpinning human behavior.

These results were published in **Science (Schmidt et al., 2009)**.

Neural bases of genetically-determined vulnerability to sleep loss

Cognition is regulated across the 24-h sleep-wake cycle by circadian rhythmicity and sleep homeostasis through unknown brain mechanisms. We investigated these mechanisms in an fMRI study of executive-function during a normal sleep-wake cycle and during sleep-loss. The study population was stratified according to homozygosity for a variable-number (*4* or *5*) tandem-repeat polymorphism in the coding region of the clock gene *PERIOD3 (PER3*). This polymorphism confers vulnerability to sleep-loss and circadian misalignment through its effects on sleep homeostasis. In the less-vulnerable genotype, no changes were observed in brain responses during the normal-sleep wake cycle. During sleep-loss, these individuals recruited supplemental anterior frontal, temporal and subcortical regions and thalamo-prefrontal connectivity was enhanced, while executive function was maintained. By contrast, in the vulnerable genotype, activation in a posterior prefrontal area was already reduced when comparing the evening to the morning during a normal sleep-wake cycle. Furthermore, in the morning after a night of sleep-loss, widespread reductions in activation in prefrontal, temporal, parietal and occipital areas were observed in this genotype. These differences occurred in the absence of genotype-dependent differences in circadian phase. The data show that dynamic changes in brain responses to an executive-task evolve across the sleep-wake and circadian cycles in a regionally-specific manner that is determined by a polymorphism which affects sleep homeostasis. The findings support a model of individual differences in executive control, in which the allocation of prefrontal resources through thalamic activation is constrained by sleep pressure and circadian phase.

These results were published in the **Journal of Neuroscience (Vandewalle et al., 2009b)**.

Non classical photoreception

Light therapy is an effective treatment for mood disorders. Here we asked whether exposure to light influences emotional brain function. During functional magnetic resonance imaging, healthy volunteers listened to emotional and neutral voices while being exposed to alternating 40s-periods of blue or green ambient light. Blue (relative to green) light increased responses to emotional stimuli in the voice area of the temporal cortex and in the hippocampus. During emotional processing, the functional connectivity between the voice area, the amygdala and the hypothalamus was selectively enhanced in the context of blue illumination. These results demonstrate the acute influence of light on emotional brain processing and identify a network merging affective and irradiance/light information. The superiority of blue over green light suggests the involvement of melanopsin-dependent photoreception in emotion regulation.

These results are now **submitted for publication**.

Our work about the influence of non classical photoreception on the neural correlates of cognition were recently summarized in a review paper published in **Trends of Cognitive Sciences (Vandewalle et al., 2009a)**.

Humans are a diurnal species usually exposed to light while engaged in cognitive tasks. Light not only guides performance on these tasks through vision but also exerts non-visual effects which are mediated in part by recently discovered retinal ganglion cells maximally sensitive to blue light. We review recent neuroimaging studies which demonstrate that the wavelength, duration and intensity of light exposure modulates brain responses to (non-visual) cognitive tasks. These responses to light are initially observed in alertness-related subcortical structures (thalamus, hypothalamus, brainstem) and limbic areas (amygdala and hippocampus), followed by modulations of activity in cortical areas, which can ultimately affect behaviour. Light emerges as an important modulator of brain function and cognition.

Sound processing during non REM sleep

Humans become less responsive to the environment during sleep. However, the extent to which the human brain responds to external stimuli during sleep is uncertain. We used simultaneous electroencephalography (EEG) and functional magnetic resonance imaging (fMRI) to characterize brain responses to tones during wakefulness or non rapid eye movement (NREM) sleep, in the presence or absence of a sleep spindle. Sounds during wakefulness elicited responses in the thalamus and primary auditory cortex. These responses persisted during NREM sleep, except during spindles during which they became much less consistent. In a computational model of neural thalamo-cortical activity, burst firing in thalamic cells during NREM sleep hindered the faithful transmission of afferent inputs, especially during spindles. Although the brain still processes information from the environment during NREM sleep, the distortion of sensory information at the thalamic level, especially during spindles, might provide unique conditions favorable for offline memory processing.

These results are now **submitted for publication**.

Effects of sleep and lack of sleep on remote memories

Sleep promotes memory consolidation, a process by which fresh and labile memories are reorganized into stable memories. Emotional memories are usually better remembered than neutral ones, even at long retention delays. In this study, we assessed the influence of sleep during the night following encoding onto the neural correlates of recollection of emotional memories six months later. After incidental encoding of emotional and neutral pictures, half of the subjects were allowed to sleep, whereas the others were totally sleep deprived, on the first post-encoding night. During subsequent retest fMRI sessions taking place three days and six months later, subjects made recognition memory judgments about the previously studied and new pictures. Between these retest sessions, all participants slept as usual at home. At six-month retest, recollection was associated with significantly larger responses in subjects allowed to sleep than in sleep deprived subjects, in the ventral medial prefrontal cortex (vMPFC) and the precuneus, two areas involved in memory retrieval, as well as in the amygdala and the occipital cortex, two regions the response of which was modulated by emotion at encoding. Moreover, the functional connectivity was enhanced between the vMPFC and the precuneus, as well as between the amygdala, the vMPFC and the occipital cortex in the sleep group relative to the sleep-deprived group. These results suggest that sleep during the first post-encoding night profoundly influences the long-term systems-level consolidation of emotional memory and modifies the functional segregation and integration associated with recollection in the long term.

These results were published in the **Journal of Neuroscience (Sterpenich et al., 2009)**.

Does sleep promotes false memories ?

Memory is constructive in nature so that it may sometimes lead to the retrieval of distorted or illusory information. Sleep facilitates accurate declarative memory consolidation but might also promote such memory distortions. We examined the influence of sleep and lack of sleep on the cerebral correlates of accurate and false recollections using functional magnetic resonance imaging (fMRI). After encoding lists of semantically related word associates, half of the participants were allowed to sleep, whereas the others were totally sleep deprived on the first post-encoding night. During a subsequent retest fMRI session taking place three days later, participants made recognition memory judgments about the previously studied associates, critical theme words (which had not been previously presented during encoding) and new words unrelated to the studied items. Sleep, relative to sleep deprivation, enhanced accurate and false recollections. No significant difference was observed in brain responses to false or illusory recollection between sleep and sleep deprivation conditions. However, after sleep but not after sleep deprivation (exclusive masking), accurate and illusory recollections were both associated with responses in the hippocampus and retrosplenial cortex. The data suggest that sleep does not selectively enhance illusory memories but rather tends to promote systems-level consolidation in hippocampo-neocortical circuits of memories subsequently associated with both accurate and illusory recollections. We further observed that during encoding, hippocampal responses were selectively larger for items subsequently accurately retrieved, than for material leading to illusory memories. The data indicate that the early organization of memory during encoding is a major factor influencing subsequent production of accurate or false memories.

These results were under the press in the **Journal of Cognitive Neuroscience (Darsaud et al., under the press)**.

Attention deficits in major depression

The pathophysiology of major depressive disorder (MDD) includes both affective and cognitive dysfunctions. We aimed to clarify how regions regulating affective processing interact with those involved in attention, and how such interaction impacts on perceptual processing within sensory cortices. Based on previous work showing that top-down influences from attention can determine the processing of external inputs within early sensory cortices, we tested with functional MRI (fMRI) whether MDD alters attentional ('top-down') effects on the neural filtering of irrelevant, non-emotional visual stimuli. The present fMRI study was conducted in 14 non-medicated patients with a first episode of unipolar MDD and 14 matched controls. During scanning, subjects performed two tasks imposing two different levels of attentional load at fixation (easy or difficult), while irrelevant colored stimuli were presented in the periphery. Analyses of fMRI data revealed that MDD patients show (i) an abnormal filtering of irrelevant information in visual cortex, (ii) an altered functional connectivity between fronto-parietal networks and visual cortices, and (iii) a hyperactivity in subgenual cingulate/medial orbitofrontal cortex that was modulated by attentional load. These results demonstrate that biological abnormalities contribute to the cognitive deficits seen in major depression, and clarify how neural networks implicated in mood regulation influence executive control and perceptual processes. These findings do not only improve our understanding of the pathophysiological mechanisms underlying cognitive dysfunctions in MDD, but also shed new light on the interaction between cognition and mood regulation.

These results were published in the **Journal of Neuroscience (Desseilles et al., 2009)**.

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Exploration of the memory enhancing effects of angiotensin IV and unravelling its mechanism of action

I. Introduction

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

In this project we investigate the hexapeptide angiotensin IV (Ang IV) because of its facilitatory role in memory acquisition and retrieval. This peptide is a fragment of the cardiovascular hormone angiotensin II (Ang II) known from its effects on the regulation of sodium excretion, body fluid volumes and arterial blood pressure. While Ang IV binds with low affinity to the classical Ang II receptors (AT_1 and AT_2), there is now accumulating evidence that most of its effects are mediated via the 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 since it binds with high affinity Ang IV as well as synthetic derivatives Norleucine^{l}-Ang IV (Nle^{l}-Ang IV) and Norleucinal (Chai et al., 2000, Albiston et al., 2001). In addition, hemorphins, a class of endogenous central nervous system peptides obtained by hydrolysis of the beta chain of hemoglobin (Møeller et al., 1997), are found to bind to AT_4 receptors. Among them LVV-H7 is the most potent of these peptides. The "AT_4 receptor" has been clearly 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; Albiston et al., 2009). IRAP is a type II integral membrane protein homologous to aminopeptidase N (APN), 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

Ang IV and related AT_4 ligands have been described to display beneficiary effects in animal models for cognitive impairment and epileptic seizures (for review see De Bundel et al., 2008). These exciting findings initiated the search for their mechanism of action. The key finding of Ang IV to enhance memory acquisition and recall was reported 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).

II. Initial working hypotheses and aims of the project

(i) First we aim to clarify the function of IRAP in memory processes by comparing the effects of Ang IV in **IRAP knockout (KO) mice** with those in wild-type (WT) mice in several memory tasks (in collaboration with S. Chai and A. Albiston, Australia). Similar experiments with Ang IV administration will be performed in **transgenic mice** that manifest several characteristic behavioural and neuropathological features of Alzheimer's disease.

(ii) The second aim is to further explore by which **mechanisms of action** the Ang IV-IRAP interaction is able to trigger physiologically relevant intra- as well as extracellular processes and how its facilitates memory functioning. This approach will be initially focused on intact-cell experiments and relevant outcomes will serve as a rationale (or tools) for dedicated *in vivo* tests.

(iii) Finally, **more stable Ang IV ligands** will be designed and evaluated. We will investigate their stability and affinity for IRAP *in vitro*. The most promising compounds will then be administered *in vivo* to wild-type and Alzheimer mice and tested for their memory-promoting effects.

III. Results

III 1. Results obtained with transgenic mice

Since several years we have now a fruitful and ongoing collaboration with Anthony Albiston and Siew Chai from the University of Melbourne (Australia). Our main joint hypothesis is that the memory enhancing effects of Ang IV and LVV-H7 are due to pharmacological inhibition of IRAP. We possess a strain of mice with a targeted deletion of the IRAP gene in collaboration with these Australian colleagues. One of our team members, Dimitri De Bundel, also performed one year of experimental work in the Australian lab in the frame of this collaboration. Albiston and Chai were both co-promotor of the PhD thesis of Dimitri De Bundel who successfully defended his thesis in June 2009 at the Vrije Universiteit Brussels under the promotorship of Yvette Michotte and Ilse Smolders. During the course of 2009, we learned from Albiston and Chai that they made already a lot of progress in conducting a comprehensive analysis of the behavioural phenotype of the IRAP KO mice and in testing the memory and learning capacities of the IRAP KO mice in various tasks for memory and learning. Therefore this work package described in the initial project proposal was not repeated within the laboratory of the Vrije Universiteit Brussel. Moreover, our Australian colleagues did an unexpected finding. Inconsistent with our common hypothesis they found that permanent deletion of IRAP resulted in mice with an age-related deficit in spatial memory (Albiston et al., 2009). A significant genotype difference was detected in performance of 6 month old IRAP KO mice in the Y maze tasks but was without effect in two other memory paradigms, i.e. novel object recognition and T maze spontaneous alternation (Albiston et al., 2009). The IRAP KO mice thus experience a more rapid onset in spatial memory deficits in comparison with their wild type litter mates. It would be interesting to investigate whether similar effects would be found in an inducible knockout/ knockdown mouse. Unfortunately such a mouse strain has not yet been developed.

For the second part of this work package, i.e. to examine the effects of Ang IV in a murine model of Alzheimer's disease, we started experiments in collaboration with Prof. P.P. De Deyn and Dr. D. Van Dam of the University of Antwerp. Their APP23 model (Van Dam and De Deyn, 2006) provides a unique opportunity to asses IRAP as a target for the development of drugs for the treatment of Alzheimer's disease. Twenty six male heterozygous APP23 mice with an average age of 3 months old were used in a first set of experiments. They were all stereotaxically implanted with an injection guide cannula

aimed at the lateral ventricle since this route of administration has best been described for the memory enhancing effects of Ang IV. The mice were allowed to recover for a minimum of 7 days and were daily handled for 1 minute during this time. Then they were subjected to the standard Morris water maze protocol by researchers blinded to the genetic and treatment status of the animals. The mice received a daily injection of aCSF, 1 nmol Ang IV or 1 nmol LVV-H7 10 minutes before the start of each trial series. Acquisition training consisted of eight sessions of four daily trials (15-min intertrial interval) starting from four different positions of the circular pool in a semi-random order. Unfortunately many technical problems hampered us to draw conclusions from this study. The mice with a guide cannula in the lateral ventricle had severe difficulties with swimming and the dental cement caps to secure the injection cannulas got loose in many mice. These are also the main reasons why this pilot experiment failed. Indeed, up till now memory enhancing effects of several drugs, e.g. memantine, within the APP23 mouse model have always been shown after systemic administration (Van Dam et al., 2005).

III.2. Results obtained on the mechanism of memory promoting action

III.2.1. Glucose hypothesis

The IRAP ligands Ang IV and LVV-H7 enhance performance in a range of memory paradigms in normal rats and ameliorate memory deficits in rat models for amnesia. The mechanism by which these peptides facilitate memory remains however to be elucidated. In recent in vitro experiments, it was demonstrated that Ang IV and LVV-H7 potentiated activity-evoked glucose uptake into hippocampal neurons (Fernando et al., 2008). This raises the possibility that IRAP ligands may facilitate memory in hippocampus-dependent tasks through enhancement of hippocampal glucose uptake. This is a sound hypothesis since IRAP and the GLUT4 glucose transporter co-exist in neurons (Fernando et al., 2007). Moreover, both proteins are co-transported between intracellular stores and the cell surface. In fact, IRAP is the only protein identified that has the same insulin-regulated trafficking characteristics as GLUT4 (Keller et al., 1995; Ross et al., 1996; Sumitani et al., 1997). In adipocytes IRAP is known to be localised almost exclusively with GLUT4 in specialised vesicles (Keller et al., 1995).

This makes Ang IV a unique ligand that may interfere with IRAP recycling and by this way enhance or prolong the exposure of GLUT4 at the cell surface. The thereby increased glucose uptake could then elicit an increased learning capacity. Indeed, trials with both rodents and humans have demonstrated that glucose enhances cognitive performance. Interestingly, this is particularly the case in elderly subjects and in patients with Alzheimer's disease (review McNay and Gold, 2002).

To further elaborate on the results on hippocampal neurons, we studied the effects of Ang IV and LVV-H7 on glucose uptake in a hippocampal cell line (P40H1 cells) and adipocytes (differentiated 3T3-L1 cells by using 2-deoxy-3H-glucose). Special attention was given to the effect of insulin (leading to a higher concentration of IRAP at the cell surface) and 7B (preventing the degradation of Ang IV by APN). In both cell lines, insulin led to a concentration dependent rise in glucose uptake. As expected, this effect was pronounced in adipocytes. Although lower in hippocampal cells, this finding is in line with the little information available in the literature about the effect of insulin on GLUT4 translocation in hippocampal cells (Grillo et al., 2009). Ang IV was only able to significantly increase glucose uptake in the P40H1 cells in the presence of insulin; and 7B as well as LVV-H7 were unable tot mimic these effects. Due to the need of a large amount of independent experiments to obtain significant results, we will also study the effect of Ang IV and LVV-H7 on the translocation of IRAP by using the recombinant human IRAP tagged by the V5 epitope. Elisa experiments will hopefully shed light about the interference of Ang IV and/or LVV-H7 on IRAP recycling.

Last year we demonstrated for the first time in vivo that Ang IV and LVV-H7 also enhanced spatial working memory in the plus maze spontaneous alternation task but found no in vivo evidence for enhanced hippocampal glucose uptake or altered cerebral blood flow (De Bundel et al., 2009) (data described in detail in GSKE report 2008).

III.2.2. Involvement of the angiotensin AT_1 receptor in the memory enhancing effects of Ang IV

We thus further investigated whether other mechanisms may be involved in the central memory promoting effects of Ang IV and LVV-H7, and determined the effects of i.c.v. administration of Ang IV or LVV-H7 on hippocampal neurotransmitter levels using microdialysis in rats. Spatial working memory enhancing effects of IRAP ligands are typically observed within 30 min following their i.c.v. administration (De Bundel et al., 2009). Ang IV (1-10 nmol) and LVV-H7 (0.1-1 nmol) did not alter hippocampal dopamine, serotonin, GABA or acetylcholine levels within this time frame, suggesting that modulation of these neurotransmitters was not involved in the spatial working memory effects of Ang IV and LVV-H7. A clear and sustained decrease in hippocampal acetylcholine levels was however observed 60-100 min following i.c.v. injection of Ang IV. While increased hippocampal acetylcholine are known to be required for encoding of spatial information, decreased hippocampal acetylcholine has been proposed to be required for consolidation and retrieval of spatial information (Hasselmo, 2006).

Interestingly, our experiments also revealed that Ang IV modulated hippocampal acetylcholine levels, whereas LVV-H7 did not. Given that both Ang IV and LVV-H7 are potent competitive inhibitors of IRAP (Lew et al., 2003; Demaegdt et al., 2004), this discrepancy suggests that Ang IV may exert its effect on hippocampal acetylcholine through a binding site different from IRAP. The AT_1 receptor was a strong candidate since we previously reported that this receptor mediates pressor effects following i.c.v. administration of Ang IV (Yang et al., 2008). We next demonstrated that LVV-H7 does not bind to the AT_1 receptor in contrast to Ang IV which is a low affinity agonist of the AT_1 receptor. The observation that the potent and selective AT_1 receptor antagonist candesartan reversed the effect of Ang IV on hippocampal acetylcholine prompted us to investigate the involvement of the AT_1 receptor in the effect of Ang IV on spatial working memory in the plus maze spontaneous alternation task. And indeed, pretreatment of the rats with candesartan also abolished the spatial working memory enhancing effect of Ang IV. However, the AT_1 receptor was clearly not involved in the spatial memory facilitating effect of LVV-H7.

Following i.c.v. administration, ^{123}I-Ang IV did not diffuse to the hippocampus, suggesting an extrahippocampal site of Ang IV-mediated action. The obtained orbital pinhole SPECT image suggested that ^{123}I-Ang IV and/or its radioactive degradation products are retained around the site of administration and did not show a homogenous distribution throughout the brain.

In conclusion, we demonstrated that the AT_1 receptor is involved in the effects of Ang IV on hippocampal acetylcholine levels and spatial working memory (De Bundel et al., Journal of Neurochemistry in press, December 17th, EPub ahead of print). This paves the way for the AT_1 receptor to be involved in other cognitive effects of Ang IV as well. This does however not exclude that Ang IV mediates other central effects independently of AT_1 receptor activation and potentially through IRAP binding. In this context it has indeed been demonstrated that Ang IV protects rats against limbic seizures (Stragier et al., 2006) and experimental ischaemic stroke (Faure et al., 2006) independently of AT_1 receptor activation. Furthermore, the present findings do not exclude a potential role of IRAP as a target for memory enhancing drugs. Indeed, LVV-H7 has no affinity for the AT_1 receptor but nevertheless enhanced spatial working memory (De Bundel et al., 2009), spatial reference memory (Albiston et al., 2004) and passive avoidance memory (Albiston et al., 2004). Moreover, a newly synthesized inhibitor of IRAP that showed

no affinity for the AT_1 receptor, HFI419, also enhanced object recognition and spatial working memory (Albiston et al., 2008).

III.3. Results obtained with novel Ang IV ligands

An important handicap for the studies dealing with the physiological role of Ang IV is its rapid degradation by different proteases. Hence, there is a need for metabolically stabilized Ang IV analogues. Especially for the studies that are focused on IRAP, these analogues should also preferably display pronounced selectivity for this enzyme, not only versus AP-N but also versus the AT_1-type receptors for Ang II. We therefore started collaboration a few years ago with two chemistry departments (Prof. D. Tourwe – DSCH, VUB and Prof. M. Hallberg - Uppsala, Sweden). When producing these new analogues of Ang IV, two strategies were followed.

In the first strategy, the final goal is to prepare metabolically stable low-molecular weight ligands with the ability to cross the blood-brain barrier. Such ligands could serve as leads in lead optimization programs. To gain information on the bioactive conformation(s) that Ang IV adopts when binding to its receptor, a series of constrained Ang IV analogues starting from cyclic disulfide ring systems was prepared (2 and 4 from Axen et al., 2007, Table 1). Furthermore, analogues encompassing a 4-hydroxydiphenylmethane scaffold replacing Tyr(2) and a phenylacetic or benzoic acid moiety replacing His(4)-Pro(5)-Phe(6) were prepared. The best Ang IV mimetics in the series were approximately 20 times less potent than Ang IV as IRAP inhibitors (ex. 11 from Andersson et al., 2008, Table 1). Although the best compounds are still less potent than Ang IV, it is notable that they comprise only two amino acid residues and are considerably less peptidic in character than the majority of the Ang IV analogues previously reported as IRAP inhibitors in the literature. New compounds combining information of the previous two sets were prepared and will be tested shortly.

For the second strategy, a scan of Ang IV was performed in which the amino acids were systematically replaced by several amino acid analogues. In a first publication both β^2- and β^3-homo-amino acids were used. In general terms, the incorporation of β-homo-amino acids has been used to create peptidomimetics that not only retain biological activity, but that are also resistant to proteolysis. The combination of 2 interesting analogues (AL-5 and 6a) into one, led to AL-11 (Table 1), a stable and specific towards IRAP vs APN and AT1, analogue. As mentioned in our previous report, this compound was tritiated and the radioligand showed the same properties on endogenous and recombinant IRAP in membranes as [^{3}H]Ang IV. Because of its stability (no need of using chelators) and specificity, we can now measure binding to only IRAP in a physiologically relevant way. This allowed us, for the first time, to perform intact cell binding experiments. Due to its stability and very low extent of non-specific binding, this radioligand is highly successful for this purpose. This now gives us the opportunity to study the translocation to the cell surface and internalization of IRAP by using radioligand binding. In a second series of analogues the histidine residue in Ang IV was replaced by various conformationally constrained amino acids. The substitution of the His(4)-Pro(5) dipeptide sequence by the constrained Trp analogue Aia-Gly, in combination with beta(2)hVal substitution at the N-terminus, provided a new stable analogue AL-40 with even better properties (higher affinity for IRAP) than AL-11. Precursors for the tritiation of this analogue are presently prepared.

The best compounds of both strategies, given in Table 1 (and to be completed), will then be used in both in vitro and in vivo functional experiments to determine their agonistic/antagonistic properties in the mentioned effects of Ang IV.

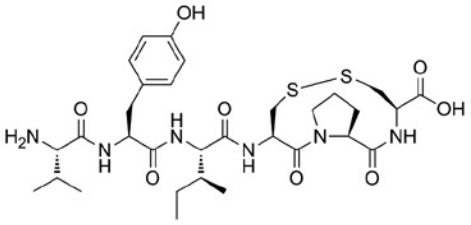
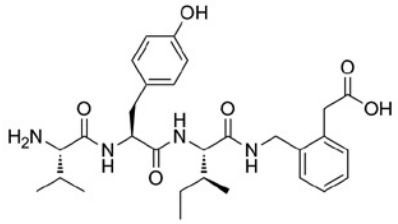
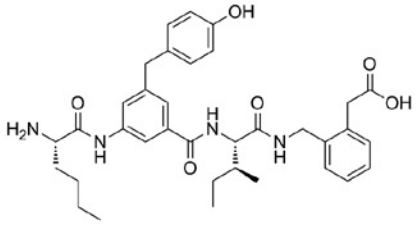
		Enzyme activity		Competition binding with labeled Ang IV/ Stability		AT1?
Compound	**Name**	**HEK293 + IRAP**	**Selective vs APN**	**Preinc. + P/E**	**Shift by P/E**	
H-Val-Tyr-Ile-His-Pro-Phe-OH	Ang IV	7.25	1.17	8.10	1.68	Yes
Low molecular weight						
	2	7.6	0.94	7.92	0.67	No
	4	7.36	1.33	7.94	0.76	No
	11	5.87	0.99	5.99	/	No
Amino acid replacement						
H-Val-Tyr-Ile-His-Pro-β^{3}hPhe-OH	AL-5	7.69	2.08	8,24	1.57	Yes
H-(R)-β^{2}hVal-Tyr-Ile-His-Pro-Phe-OH	AL-6a	7.00	1.65	7.58	-0.06	No
H-(R)-β^{2}hVal-Tyr-Ile-His-Pro-β^{3}hPhe-OH	AL-11	7.56	2.33	7.70	-0.03	No
H-(R)-β^{2}hVal-Tyr-Ile-Aia-Gly-Phe-OH	AL-40	8.07	1.97	8.26	0.14	No

Table 1: pKi values of enzyme activity inhibition and of competition binding with labelled Ang IV in CHO-K1 membranes after preincubation in the presence or absence of EDTA and 1,10-phenanthrolin (Chelators) of the most potent compounds of both strategies. Values are the mean ± SD (SEM for Ang IV) of 2 experiments performed in duplicate (Axen et al., 2007, Lukaszuk et al., 2008, 2009, Andersson et al., 2008, in preparation).

IV. Ang IV, involvement in memory mechanisms, synaptic plasticity and epilepsy

Since especially the experiments described under III.1. could not be properly finished, we extended the aims of the current project also to another field of neuroscience in which we are very active, i.e. epilepsy. The link between memory mechanisms and epilepsy mechanisms is moreover not at all farfetched. Clinically, cognitive decline is a well-known co-morbidity of epilepsy (Hermann et al., 2008)

and epileptic seizures occur in patients with dementia at a higher prevalence than among healthy elderly individuals (Mendez and Lim, 2003). Also at the level of synaptic transmission and plasticity there are strong similarities between memory and epilepsy mechanisms. Indeed, long-term potentiation (LTP) and long-term depression (LTD) are defined as a persisting enhancement or suppression of synaptic efficacy and are posited as the underlying cellular mechanism for memory formation and extinction respectively (Royer and Pare, 2003). These forms of synaptic plasticity are strikingly similar to the synaptic rearrangements observed in the kindling model for epileptogenesis and are recognized factors in the evolution of epilepsy. Last but not least, there is a large body of evidence that the peptide of our interest, Ang IV, has cognitive enhancing properties, affects clearly synaptic efficacy in the hippocampus and has a modulatory action on seizures and epileptogenesis. We have reviewed in detail the effects of Ang IV on synaptic transmission and plasticity, learning, memory, and epileptic seizure activity (De Bundel et al., 2008).

V. Role of Ang IV in epilepsy

Next to its memory promoting properties, Ang IV is found to attenuate pentylenetetrazole-induced seizures (Tchekalarova et al., 2001). Furthermore, Ang IV showed an anti-epileptogenic effect as it not only suppressed the maintenance of the generalization phenomenon during the kindling procedure but also blocked the development of an epileptic-like state in mice (Tchekalarova et al., 2005a,b). We have previously shown that i.c.v. administered Ang IV is anticonvulsant in the acute pilocarpine model for focal epilepsy in rats (Stragier et al., 2006). This was accompanied by a concomitant increase of the hippocampal extracellular dopamine and serotonin concentration. Possibly, this plays a role in the anticonvulsant effect of Ang IV. Indeed, several well-known anti-epileptic drugs can elicit a monoaminergic stimulation. Moreover, it was shown in our laboratory that intrahippocampally administered dopamine and serotonin protect rats against pilocarpine induced convulsions via respectively D_2 and 5-HT_{1A} receptor activation (Clinckers et al., 2004). Moreover, the anticonvulsant effect of Ang IV could be reversed by somatostatin sst2 receptor antagonism (Stragier et al., 2006). These results suggest that Ang IV may exert an anticonvulsive effect via inhibition of IRAP, resulting in an increase of the anticonvulsive neuropeptide somatostatin-14. Besides somatostatin, other IRAP neuropeptide substrates might also be involved in protection from seizures. IRAP is indeed an aminopeptidase known to metabolize several substrate neuropeptides in vitro, including somatostatin, oxytocin, vasopressin, lys-bradykinin, met-enkephalin, dynorphin A 1-8, neurokinin A, neuromedin B and cholecystokinin 8 ((Rogi et al., 1996; Lew et al., 2003). It was therefore suggested that Ang IV may mediate its central effects through inhibition of IRAP and accumulation of its substrates, i.e. the so-called neuropeptide hypothesis.

VI. Extended working hypotheses and aims of the project

(i) First we aim to clarify the function of IRAP in seizure mechanisms by comparing the threshold for epileptic seizures in **IRAP knockout (KO) mice** and their wild-type (WT) litter mates in several chemoconvulsant seizure models.
(ii) The second aim is to further explore by which **mechanisms of action** the Ang IV-IRAP interaction is able to induce anticonvulsant effects.
(iii) Finally, **novel developed and stable Ang IV ligands** will be evaluated in various seizure models.

VII. Results

VII.1. Results obtained with transgenic mice

To unequivocally unravel the involvement of IRAP in seizure generation, IRAP KO mice and their WT littermates were subjected to an intravenous tail infusion of pentylenetetrazole, which is an established acute model of generalised seizures. Compared to male WT mice, male KO mice showed significantly increased pentylenetetrazole thresholds for myoclonic twitch, clonus without loss of reflexes and clonus with loss of reflexes. We also tested the IRAP KO mice in a model for limbic seizures, i.e. the pilocarpine tail infusion model. IRAP KO mice had a significantly higher pilocarpine threshold compared to WT animals for the first onset of tremor, clonic convulsions, tonic convulsions and death. These data again unequivocally show that mice lacking functional IRAP are partially protected against pilocarpine-induced seizures and toxicity. In conclusion, IRAP is thus clearly involved in seizure generation, since male IRAP KO mice are less sensitive to the development of generalized seizures following pentylenetetrazole administration or limbic seizures following pilocarpine administration. Unfortunately, the mechanism by which IRAP inhibition leads to these anticonvulsive effects remains elusive.

VII.2. Results obtained on the mechanism of anticonvulsive action

Since we clarified the involvement of IRAP in seizure mechanisms, we subsequently investigated the local effects of two peptide inhibitors of IRAP, Ang IV and LVV-H7, on seizures evoked by intrahippocampal pilocarpine administration in rats. Intrahippocampal administration of Ang IV or LVV-H7 protected rats against pilocarpine induced seizures. We clearly excluded the involvement of other potential binding sites, essentially the angiotensin AT_1 receptor for Ang IV and the opioid μ/κ receptor for LVV-H7. We demonstrated that the anticonvulsive effects of both locally applied Ang IV and LVV-H7 are reversed by the sst_2 receptor antagonist cyanamid 154806. Intrahippocampal administration of somatostatin was anticonvulsive in the same model. We then hypothesized that the anticonvulsive effects of Ang IV and LVV-H7 would result from inhibition of somatostatin degradation via IRAP. We therefore initiated collaboration with Prof. Kiki Thermos of the University of Heraklion (Greece). One investigator of our team performed the necessary microdialysis experiments in the laboratory of Heraklion and the samples were analysed for somatostatin content by a validated radioimmunoassay. Nevertheless, Ang IV and LVV-H7 did not increase the extracellular somatostatin levels as such and even suppressed high potassium-evoked somatostatin release in the hippocampus of freely moving rats. Moreover, we observed no differences in the degradation of somatostatin in a cerebral membrane preparation from IRAP KO compared to WT mice. The degradation profile of labelled somatostatin in these homogenates was determined at different time intervals (0, 30, 60, 120 and 180 minutes) by an in-house validated radioimmunoassay. Taken together, all these experiments strongly suggest that the effects of Ang IV and LVV-H7 do not result from accumulation of somatostatin due to inhibition of the catalytic domain of IRAP and thus at the moment we reject the so-called neuropeptide hypothesis as a mechanism of anticonvulsive action of the IRAP ligands. We therefore propose that inhibition of IRAP may directly affect sst_2 receptor signalling and/or trafficking.

At the moment we are investigating this new hypothesis in more detail with ex vivo experiments. We posses a CHO cell line with stable expression of the sst2 receptor. We already demonstrated that this cell line has a clear endogenous expression of IRAP and thus this cell line is an ideal tool to unravel further the mechanism of action. We also excluded direct binding of Ang IV and LVV-H7 to sst_2 receptors within a well validated radioligand binding assay. In a next step of experiments we will incubate the cells with IRAP ligands for different incubation periods (1h, 6h, 24h) and we will investigate whether the sst2 receptor binding/expression will vary under these different conditions.

We can also exclude the involvement of oxytocin in the mechanism of anticonvulsant action of Ang IV within the pentylenetetrazole model. To date, the role of the IRAP substrate oxytocin in seizure mechanisms has not been well described in literature. We demonstrated recently that oxytocin has proconvulsant effects within the pentylenetetrazole model for generalised seizures. Indeed, oxytocin at a dose of 0.25 mg/kg, which we demonstrated in another set of experiments to possess antidepressant-like effects within the forced swim test and which is thus centrally active at this dose, significantly lowered the seizure threshold for ear twitch, myoclonic twitch and forelimb clonus in mice.

Finally, we can report that, in the frame of the experiments we performed in collaboration with Prof. Kiki Thermos of the University of Heraklion, we demonstrated that another somatostatin receptor subtype, the sst_1 receptor, is not involved in the anticonvulsive action of somatostatin and thus most probably also not in the mechanism of action of IRAP ligands. Interestingly, this nice set of experiments pointed for the first time in vivo that the hippocampal sst_1 receptors act as inhibitory autoreceptors. Indeed, intrahippocampal administration of the sst_1 receptor antagonist SRA880 led to a robust, but transient increase in hippocampal somatostatin levels without affecting the GABA levels. It is well known that somatostatin is a co-transmitter of the hippocampal GABAergic interneurones. Our data demonstrate that the observed effects on hippocampal somatostatin levels did not result from increased phasic firing of somatostatin-containing interneurons, but rather involved a specific loss of negative feedback control on tonic somatostatin release (De Bundel et al., NeuroReport, in press).

VIII. Publication list of 2009

1. De Bundel D, Smolders I, Yang R, Albiston AL, Michotte Y, Chai SY.
   Angiotensin IV and LVV-haemorphin 7 enhance spatial working memory in rats: effects on hippocampal glucose levels and blood flow.
   Neurobiol Learn Mem. 2009; 92(1):19-26.
   SCI impactfactor = 3.8.
2. De Bundel D, Demaegdt H, Lahoutte T, Caveliers V, Kersemans K, Ceulemans AG, Vauquelin G, Clinckers R, Vanderheyden P, Michotte Y, Smolders I.
   Involvement of the AT1 receptor subtype in the effects of angiotensin IV and LVV-Haemorphin 7 on hippocampal neurotransmitter levels and spatial working memory.
   J Neurochem. 2009; Dec 17. [Epub ahead of print]
   SCI impactfactor = 4.5.
3. De Bundel D, Aourz N, Kiagiadaki F, Clinckers R, Hoyer D, Kastellakis A, Michotte Y, Thermos K, Smolders I.
   Hippocampal sst1 receptors are autoreceptors and do not affect seizures in rats
   NeuroReport, in press.
   SCI impactfactor = 1.9.
4. Demaegdt, H, Lukaszuk, A, De Buyser, E, De Backer, JP, Szemenyei, E, Tóth, G, Chakravarthy, S, Panicker, P, Michotte, Y, Tourwé, D, Vauquelin, G.
   Selective labeling of IRAP by the tritiated AT_4 receptor ligands [^{3}H]Angiotensin IV and its stable analog [^{3}H]AL-11.
   Mol. Cell. Endocrinol, 2009; 311, 77-86.
   SCI impact Factor: 3.6
5. Lukaszuk, A, Demaegdt, H, Feytens, D, Vanderheyden, P, Vauquelin, G, Tourwé, D.
   The replacement of His(4) in angiotensin IV by conformationally constrained residues provides highly potent and selective analogues.
   J Med Chem, 2009; 52, 5612-8.
   SCI impact Factor: 4.9

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Characterization of new cellular and molecular mechanisms underlying the migration of interneurons in the telencephalon

Backround

The cerebral cortex contains neurons that are distributed within layers and are regionally organized into specialized areas that underlie sophisticated motor, cognitive and perceptual abilities. Cortical lamination follows an «inside-out» sequence of neuronal placement and maturation that arises from the successive birth and orderly migration of pyramidal projection neurons born in the dorsal telencephalon and GABAergic interneurons generated in the ganglionic eminences (GE)(Marin and Rubenstein, 2003).

Experimental observations show that most projection neurons migrate radially within the cortical anlage, whereas interneurons migrate from the GE through multiple tangential pathways to reach the developing cortex. Recent studies suggest that defects in neuronal migration may lead to several impairments, which, in human, are characterised, by learning disabilities, mental retardation or epilepsy (Levitt et al., 2004; Pancoast et al., 2005). Moreover, converging experimental and clinical evidence suggests that altered interneuron development may underlie part of the pathophysiological processes that ultimately lead to bipolar disorder, schizophrenia and autism (Benes and Berretta, 2001; Levitt et al., 2004). Defining how cortical neurons migrate and integrate into specific circuits is, therefore, essential for understanding the biological basis of these disorders.

The following report summarizes the work performed the past year thanks to the generous funding from the FMRE/GSKE and provides the perspectives of our future research.

Unravelling the functions of Cip/Kip proteins during the migration and differentiation of cortical interneurons

The generation of cortical interneurons is a complex process that relies on the decision of ventral progenitors to leave the cell cycle, migrate tangentially to appropriate locations and differentiate into mature neurons that are stably positioned and are actively extending axonal and dendrite branches. Importantly, these concurrent steps imply dynamic cell shape remodelling which largely depends on the regulation of cytoskeleton components. Our current experiments are aimed at identifying key molecules that control cytoskeleton remodelling during the migration and branching of cortical interneurons.

First identified as cell cycle inhibitors mediating the growth inhibitory cues of upstream signalling pathways, the cyclin-CDK inhibitors of the Cip/Kip family have emerged as multifonctional proteins with roles extending beyond the cell cycle regulation. A prime example is $p27^{Kip1}$ as reported by our previous work. It promotes both neuronal differentiation and migration of cortical projection neurons through distinct and separable cell-cycle independant mechanisms (Nguyen et al., 2006). Our recent analyses performed on embryonic brains revealed the expression of $p27^{Kip1}$ in progenitors located in the subventricular zone of the medial (MGE) and caudal ganglionic eminence (CGE) as well as in postmitotic cortical interneurons. Interestingly, $p27^{Kip1}$ was expressed in both the nucleus and the cytoplasm of these neurons, suggesting that it could play cell cycle-unrelated functions. In order to test this hypothesis we

analysed the generation and migration of interneurons in the cortex of p27 knockout mice (Fero et al., 1996; Nakayama et al., 1996). Surprisingly, the lack of p27 expression did not impair the proliferation nor the cell cycle exit of GE interneurons progenitors, suggesting that $p57^{Kip2}$ or $p21^{Cip1}$ might compensate for the lack of $p27^{Kip1}$. However, we found a reduced number of Lhx6-positive interneurons in the lateral cortex of p27 knockout E14 embryos that suggests a defect in tangential migration. To test this hypothesis we conditionally removed p27 in Dlx5,6-positive cortical interneurons (generated by Dlx5,6 Cre-IRES-GFP (Stenman et al., 2003) X $p27^{lox/lox}$ (Chien et al., 2006) breedings) and confirmed defects in tangential migration. We performed time lapse experiments on cultured slices from E12 embryos from similar breedings and observed a significant reduction of the speed of tangential migration when p27 was conditionally removed (33.0 +/- 1.9 μm/hour, n=46 cells versus 45.9 + /- 1.6 μm/hour, n=59 for control). These data prompt us to analyse the detailed morphology of GABAergic interneurons during their migration out of the MGE. For this purpose we cultured MGE explants from transgenic mice on wild type cortical feeders and carefully recorded the front of migration using time-lapse analyses. Cells that lack p27 were undergoing unusualy quick but less efficient nucleokinesis, as the total distance travelled by the nucleus was reduced. In addition, the absence of p27 resulted in an uncoordinated production of branches on the leading process. These cellular defects likely account for the reduced migration speed of the GABAergic interneurons that are invading the cerebral cortex of Dlx5,6 Cre-IRES-GFP ; $p27^{lox/lox}$ embryos in vivo.In addition, we demonstrated that p27 regulates the tangential migration of interneurons through cell cycle-independent activities as its acute knockdown by electroporation-based transfection of shRNAs in postmitotic interneurons resulted in defective tangential migration and, cortical interneurons generated in $p27^{ck-}$ embryos (knock-in mouse where the coding sequence of $p27^{Kip1}$ has been swapped with a mutant version of $p27^{Kip1}$ ($p27^{ck-}$) that cannot promote cell cycle exit ; (Besson et al., 2004; Nguyen et al., 2006) did not show obvious migration defects.

We are currently analysing the molecular pathways triggered by $p27^{Kip1}$ that controls the nucleokinesis and the dyamic branching of cortical interneurons during tangential migration. Furthermore, we plan to assess whether $p27^{Kip1}$ also contributes to the regulation of terminal differentiation and fate specification of cortical interneurons. Surprisingly, our preliminary results suggest that the terminal branching of interneurons (growth of dendrites and axones) that lack p27 is poorly affected. Finally, we will perform some immunostainings (calbindin, somatostatin, paravalbumin,..) on brain from postnatal mice generated from Dlx5,6:Cre-IRES-GFP X $p27^{lox/lox}$ breedings and analyse neuronal specification by confocal microscopy.

Defining Elongator functions during the migration and differentiation of cortical interneurons

The dynamic remodelling of the cytoskeleton provides the driving force required for cell migration. Several molecules that interact with either actin filaments or microtubules have been identified as part of the molecular machinery that underlie the radial migration of projection neurons. We have recently identified the Elongator complex as a new element of this machinery.

Elongator is composed by 6 subunits (Elp1-Elp6) and assembled by its scaffold protein Elp1 (Close et al., 2006; Petrakis et al., 2004). Elp3 is the catalytic subunit which acetylates histone H3 (Hawkes et al., 2002; Kim et al., 2002; Winkler et al., 2002). Elongator associates with several nascent RNAs in yeast (Kristjuhan and Svejstrup, 2004) and is preferentially recruited to the transcribed regions of

human genes (Close et al., 2006; Kouskouti and Talianidis, 2005), which supports a role in transcript elongation. Other reports also provided evidences for a role in exocytosis and tRNA modification in the cytoplasm (Esberg et al., 2006; Huang et al., 2005; Rahl et al., 2005). Elongator deficiency in humans causes familial dysautonomia (FD), an autosomal recessive disease characterized by defects in the development and maintenance of neurons of the autonomic and sensory systems (Axelrod, 2004; Slaugenhaupt and Gusella, 2002). While neuropathological reports have mostly described lesions in the peripheral nervous system (PNS) (Pearson and Pytel, 1978; Pearson et al., 1978), functional neuroimaging analyses supported central defects with unusual activities in specific cortical areas (Axelrod et al., 2000). In addition, we showed that the depletion of Elongator cell-autonomously delayed radial migration and impaired branching of cortical projection neurons by reducing the acetylation of α tubulin (Creppe et al., 2009).

Our current results show that Elongator subunits are also expressed in the GE, where cortical GABAergic interneurons are generated. Thus, we will assess if Elongator takes part to the regulation of the migration and branching of cortical interneurons. For this purpose, we have generated an $Elp3^{lox/lox}$ transgenic mouse line and received another line $Elp1^{lox/lox}$ (KOMP repository) that will be used for conditional removal of Elp3 and Elp1 in Dlx5,6:Cre-IRES-GFP interneurons. This strategy will allow us to analyse the generation, migration and branching of Elongator deficient cortical interneurons.

We recently identified α tubulin as the first cytoplamic target of Elp3. Its proper acetylation by Elp3 is required for the regulation of both, branching and migration of cortical projection neurons (Creppe et al., 2009). While a variety of cytoplasmic and mitochondrial proteins are known to be acetylated, the role of such modification and the identity of the acetylase often remains unknown (Kim et al., 2006). We are currently characterizing other relevant cytoplasmic substrates of Elp3 that can be acetylated and as such underlie neurogenesis in the brain. To unravel this issue, we are following two experimental approaches. First, in order to characterise the Elp3-dependent acetylome (all proteins that undergo acetylation in the cytoplasm) of the developing cortex, we will use a conditional gene knockout approach ($Elp3^{lox/lox}$; FoxG1:Cre) to prevent the expression of Elp3 in cortical projection neurons. Dorsal or ventral progenitors and newborn neurons will be isolated prior or after *crelox*-mediated invalidation and then processed by nano high-performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) analyses to characterize the acetylome and uncover specific non-core histone targets of Elp3. We will follow a complementary approach to validate the acetylation of proteins that have recently been described in large acetylome screens (Choudhary et al., 2009; Kim et al., 2006) and that promote brain neurogenesis. Among them, Filamin A has recently been shown to bind Elp1, an interaction required to target filamin A to membrane ruffles and thus promotes neuron migration (Johansen et al., 2008). As such, Filamin A is a strong acetylation target of Elp3. To confirm this hypothesis, we will look for filamin A acetylation in cortical neurons isolated from $Elp3^{lox/lox}$; FoxG1:Cre mouse embryos (E14) and analyse the morphology and migration of cortical projection neurons expressing key acetylated K-to-R filamin A proteins. We will also assess if filamin A is a direct target of Elp3 by performing *in vitro* acetylation assays (Creppe et al., 2009).

The remaining candidate proteins identified by MS-based quantitative proteomics will be validated by *in vitro* acetylation assays (Creppe et al., 2009) and the biological significance of their modification will be experimentally addressed *in vivo*. Their expression pattern will be analysed by immunohistochemistry when commercial antibodies are available or by *in situ* hybridization. The candidates showing the most promising expression pattern regarding the regulation of key developmental steps in corticogenesis (proliferation, migration and differentiation), will be analysed in more details. Thus, the endogenous

candidate proteins will be silenced using shRNA encoding plasmids and replaced by shRNA refractory mutant protein harbouring key acetylated K-to-R mutations. The resulting phenotype will be analysed by immunohistochemistry.

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Characterization of G protein-coupled receptors involved in drug addiction and motor diseases

1. The GPR3 receptor in emotional-like responses

GPR3 is an orphan G-protein-coupled receptor (GPCR) which, upon transfection in various mammalian cell lines, causes strong constitutive activation of adenylyl cyclase, in the absence of any added agonist. In mouse oocytes, GPR3 contributes to maintenance of cAMP concentrations at a level required to ensure meiotic arrest in prophase I until the LH surge. Whether cAMP accumulation is the result of a true constitutive activity of the receptor or the consequence of the chronic stimulation by a ubiquitous unknown ligand, is still debated. Sphingosine 1-phoshate was proposed as an agonist of the rat GPR3 homologue but this has not been confirmed yet. GPR3 transcripts are also widely expressed in the mouse brain, in areas related to different physiological functions. More specifically, GPR3 receptor is expressed in the main brain structures involved in stress-related behaviors such as habenula but also hippocampus, amygdala, limbic system and cortex. Interestingly, the highest levels of expression were found in the habenula. The habenular complex is an important relay station between the limbic forebrain and the midbrain. It has been clearly shown to participate in the regulation of ascending monoamine and acetylcholine transmission towards hippocampus and frontal cortex. Serotonin and noradrenaline systems are known to play a role in the regulation of several central activities including mood and anxiety. Dysregulation of these systems appears to have a role in the pathophysiology of depression and anxiety disorders. Given the ability of GPR3 to activate the cAMP regulatory cascade in a tonic way in areas involved in stress-related behaviors, we hypothesized that GPR3 could play a role in the control of the corresponding behavioral responses.

We investigated therefore the consequences of genetic deletion of GPR3 in several behavioral paradigms and on neurotransmission. Compared to wild-type, hippocampal neurons from Gpr3$^{-/-}$ mice displayed lower basal intracellular cAMP levels, consistent with the strong constitutive activity of GPR3 in transiently transfected cells. Gpr3$^{-/-}$ mice developed and behaved normally with neither major changes in locomotion under basal conditions nor impairment in motor coordination. No deficits in avoidance learning were evidenced in Gpr3$^{-/-}$ mice, which exhibited similar performance than wild-type mice in the active avoidance paradigm. This suggests that the lack of GPR3 does not affect the learning of fear which primarily develops through a conditioned process. However, Gpr3$^{-/-}$ mice exhibited a higher level of anxiety-related responses after exposure to unfamiliar stressful environment in the open-field and the elevated plusmaze paradigms, including a behavioral inhibition observed as a reduced activity in the open-field test. The anxiety-like phenotype observed in the elevated plus-maze was sensitive to the effect of benzodiazepines since the administration of diazepam reversed the anxiogenic response. In addition, an increase in the number of attacks and a decrease in the latency period for the first attack were observed in the resident-intruder test, revealing a higher level of aggressiveness in mutant mice. Thus, the behavioral phenotype argues for a possible link between emotional reactivity and aggressive behavior in mutant mice. It also suggests that Gpr3$^{-/-}$ mice could be more susceptible to develop a ''depression-related'' behavior when exposed to a stressful situation from which they cannot escape. Indeed Gpr3$^{-/-}$ mice exhibited a behavioral despair as evidenced by an increased duration of immobility in the tail suspension and the forced swim tests, which are widely used to assess the efficacy of antidepressant drugs and genetic manipulation relevant to depression. We did not notice any change in serum corticosterone levels in Gpr3$^{-/-}$ mice under basal conditions or in response to the stress induced by the tail suspension test. Therefore, the mechanism underlying the behavioral characteristics of

Gpr3$^{-/-}$ mice does not seem to be related to an alteration of the HPA axis activity.

Monoaminergic neurotransmission is thought to modulate mood states and the stress response. Important alterations of the brain monoaminergic systems have been involved in mood disorders, and animal studies proposed an inverse relationship between the activity of the brain 5-HT system and aggressive behavior. Therefore, we analyzed the tissue levels of 5-HT, norepinephrine (NE) and dopamine (DA) in several brain structures of Gpr3$^{-/-}$ mice. We found that Gpr3$^{-/-}$ mice exhibit abnormally low levels of monoamines in various brain areas under basal conditions. In particular, the dramatic decrease in 5-HT content observed in hippocampus, hypothalamus and frontal cortex could well account for the behavioral despair and aggressiveness displayed by the Gpr3$^{-/-}$ mice and indicate a role for GPR3 in modulating the serotonergic system. The observation of a significant decrease in NE in cortex and hypothalamus could also account for the behavioral phenotype of knockout mice. Because the metabolites of 5-HT and DA were also decreased in Gpr3$^{-/-}$ mice, it seems likely that the primary target of the regulation by GPR3 is the synthesis or reuptake of these neurotransmitters. Conversely, in case of NE, the primary metabolite NM was found to be increased indicating that its metabolism is affected by Gpr3 deletion.

The expression of GPR3 showed the highest level in the habenula. The habenular complex is an evolutionarily conserved diencephalic structure linking the forebrain with midbrain and hindbrain structures. It has been shown to participate in the regulation of monoamine transmission. Through a habenulo-raphe pathway it modulates serotonergic activity in many structures including the hippocampus and influences the noradrenergic activity through a connexion with the locus coeruleus. Our results suggest that such a control is modulated at least in part through GPR3.

Interestingly, mice deficient in phosphodiesterase-4D (PDE-4D) and animals treated with the PDE-4 inhibitor rolipram display an attenuated despair behavior when exposed to the same behavioral models used in the present study. Since GPR3 and PDE-4D are both highly expressed in medial habenula and have antagonistic action on the intracellular cAMP level, these data strengthen the notion that basal levels of cAMP in habenular neurons are an important parameter for the emotional-like behaviors under the control of the limbic system.

This study demonstrates therefore that GPR3 plays an important role in modulating several responses in animal models consistently employed to evaluate emotional disorders including anxiety, depression-like disorders, and aggressiveness, probably by tuning the monoaminergic neurotransmission in various brain regions. In consequence, GPR3 dysfunction could be involved in the etiology of disorders associated with emotional disturbances, thereby representing a novel actor of the cAMP-dependent signaling pathway linked to behavioral responses (Valverde et al. 2009).

2. In vivo function of the adenosine A_{2A} receptor

The neuromodulator adenosine, which acts through the receptors A_1, A_{2A}, A_{2B} and A_3, has been proposed as an endogenous anticonvulsant. We investigated the consequences of deleting the adenosine A_{2A} receptor in different experimental models of epilepsy. A_{2A}R KO mice were not protected against seizures originating from brainstem structures, namely electroshock-induced seizures. The intensities of seizures induced by pentylenetetrazol or pilocarpine, as well as the percentages of convulsing mice, were

significantly reduced in A_{2A} receptor knockout (A_{2A}R KO) animals. A_{2A}R KO mice exhibited reduced pentylenetetrazol-induced kindled seizures, demonstrating an important role of the A_{2A} receptor in the acquisition of kindling. These data suggest that adenosine stimulating A_{2A} receptors modulates excitatory neurotransmission and exacerbates limbic seizures. It is therefore suggested that adenosine A_{2A} receptor antagonists might offer protection from some epileptic syndromes (El Yacoubi et al. 2009).

Adenosine A_{2A}, cannabinoid CB_1 and metabotropic glutamate 5 ($mGlu_5$) receptors are all highly expressed in the striatum. We investigated whether, and by which mechanisms, these receptors interact in the regulation of striatal synaptic transmission. By extracellular field potentials recordings in corticostriatal slices, we demonstrated that the ability of the selective CB_1 agonist WIN55,212-2 to depress synaptic transmission was prevented by the pharmacological blockade or the genetic inactivation of A_{2A}Rs. Such a permissive effect of A_{2A}Rs towards CB_1Rs does not seem to occur pre-synaptically as the ability of WIN55,212-2 to increase the R2/R1 ratio under a protocol of paired-pulse stimulation was not modified by ZM241385. Furthermore, the effects of WIN55,212-2 were reduced in slices from mice lacking post-synaptic striatal A_{2A}Rs. The selective $mGlu_5$R agonist CHPG potentiated the synaptic effects of WIN55,212-2, and such a potentiation was abolished by A_{2A}R blockade. Unlike the synaptic effects, the ability of WIN55,212-2 to prevent NMDA-induced toxicity was not influenced by ZM241385. These results show that the state of activation of A_{2A}Rs regulates the synaptic effects of CB_1Rs and that A_{2A}Rs may control CB_1 effects also indirectly, namely through $mGlu_5$Rs (Tebano et al. 2009).

Dopamine D_2 and adenosine A_{2A} receptors are highly enriched in striatal neurons and exhibit strong interactions. By performing perforated-patch-clamp recordings on brain slices, it was shown that membrane potential transitions and firing patterns in striatal neurons are tightly controlled by D_2 and A_{2A} receptors through specific protein-protein interactions including A_{2A}-D_2 receptors heteromerization (Azdad et al. 2009).

3. In vivo function of the CB_1 cannabinoid receptor

Experimental evidence indicates that endogenous cannabinoid mechanisms play important roles in nociceptive information processing in various areas of the nervous system including the spinal cord. Although it is well documented that the CB1 receptor is strongly expressed in the superficial spinal dorsal horn, its cellular distribution is poorly defined, hampering the interpretation of the effect of cannabinoids on pain processing spinal neural circuits. We investigated therefore the cellular distribution of CB_1 in laminae I and II of the rodent spinal dorsal horn by immunocytochemistry. Axonal varicosities revealed a strong immunoreactivity for CB_1, but no CB_1 expression was observed on dendrites and perikarya of neurons. Investigating the co-localization of CB_1 with markers of peptidergic and non-peptidergic primary afferents, and axon terminals of putative glutamatergic and GABAergic spinal neurons, we found that nearly half of the peptidergic (immunoreactive for calcitonin gene-related peptide) and more than 20% of the non-peptidergic (binding isolectin B4) nociceptive primary afferents, more than one-third and approximately 20% of the axon terminals of putative glutamatergic (immunoreactive for vesicular glutamate transporter 2) and GABAergic (immunoreactive for glutamic acid decarboxylase; GAD65 and/or GAD67) spinal interneurons, respectively, were positively stained for CB_1. In addition to axon terminals, almost half of the astrocytic (immunoreactive for glial fibrillary acidic protein) and nearly 80% of microglial (immunoreactive for CD11b) profiles were also immunolabeled for CB_1. These findings suggest that the activity-dependent release of endogenous cannabinoids activates a complex

signaling mechanism in pain processing spinal neural circuits into which both neurons and glial cells may contribute (Hegyi et al. 2009).

Serotonergic and endocannabinoid systems are important substrates for the control of emotional behaviour and growing evidence show an involvement in the pathophysiology of mood disorders. We showed that the absence of the activity of the CB_1 cannabinoid receptor impaired serotonergic negative feedback in mice. Thus, in vivo microdialysis experiments revealed increased basal 5-HT extracellular levels and attenuated fluoxetine-induced increase of 5-HT extracellular levels in the prefrontal cortex of CB_1 knockout compared with wild-type mice. These observations could be related to the significant reduction in the 5-HT transporter binding site density detected in frontal cortex and hippocampus of CB_1 knockout mice. The lack of CB_1 receptor also altered some 5-HT receptors related to the 5-HT feedback. Extracellular recordings in the dorsal raphe nucleus (DRN) revealed that the genetic and pharmacological blockade of CB_1 receptor induced a $5\text{-}HT_{1A}$ autoreceptor functional desensitization. In situ hybridization studies showed a reduction in the expression of the $5\text{-}HT_{2C}$ receptor within several brain areas related to the control of the emotional responses, such as the DRN, the nucleus accumbens and the paraventricular nucleus of the hypothalamus, whereas an over-expression was observed in the CA3 area of the ventral hippocampus. These results reveal that the lack of CB_1 receptor induces a facilitation of the activity of serotonergic neurons in the DRN by altering different components of the 5-HT feedback as well as an increase in 5-HT extracellular levels in the prefrontal cortex in mice (Aso et al. 2009).

The CB_1 cannabinoid receptor has also been implicated in the control of fear and anxiety. We investigated the effects of genetic and pharmacological blockade of the CB_1 cannabinoid receptor on the behaviour of CD1 mice using three different ethological models of fear and anxiety (elevated T-maze and plus-maze and open field test of emotionality). We also measured tissue levels of noradrenalin (NA), dopamine (DA), serotonin (5-HT) and their metabolites in several forebrain regions, to examine the relationship between CB_1 receptor manipulation and monoaminergic neurotransmission. The CB_1 receptor antagonist SR141617A (rimonabant) modulated anxiety in a dose-dependent manner. At a dose of 3 mg/kg i.p., the compound consistently increased anxiety parameters in the three different anxiety tests applied, while a lower dosage of 1 mg/kg had no such effect. The neurochemical evaluation of the mice administered 3mg/kg SR141617A revealed increases in the concentrations of DOPAC and 5-HIAA in the dorsal striatum, elevated DA levels in the hippocampus and reduced dopamine turnover in the septum. Furthermore, these animals had a higher HVA/DA turnover in the frontal cortex. CB_1 receptor knockout mice as well as mice treated with the selective CB_1 receptor antagonist AM251 did not display any significant alterations in anxiety-related behaviour as measured with the elevated plus-maze and open field test of emotionality, respectively. Our findings support the general idea of a SR141617A-sensitive receptive site that is different from the 'classical' CB_1 receptor and that has a pivotal role in the regulation of different psychological functions. Under physiological conditions this receptive site seems to be involved in the control of anxiolysis (Thiemann et al. 2009).

Regulation of Ca^{2+} homeostasis plays a critical role in oligodendrocyte function and survival. Cannabinoid CB_1 and CB_2 receptors have been shown to regulate Ca^{2+} levels and/or K^+ currents in a variety of cell types. We investigated the effect of cannabinoid compounds on the Ca^{2+} influx elicited in cultured oligodendrocytes by transient membrane depolarization with an elevated extracellular K^+ concentration (50 mM). The CB_1 receptor agonist arachidonoyl-chloro-ethanolamide (ACEA) elicited a concentration-dependent inhibition of depolarization-evoked Ca^{2+} transients in oligodendroglial somata. This activity was mimicked by the CB_1/CB_2 agonist CP55,940, as well as by the endocannabinoids N-arachidonoyl-

ethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), whereas the CB_2 receptor selective agonist JWH133 was ineffective. The CB_1 receptor antagonist AM251 also reduced the Ca^{2+} response evoked by high extracellular K^+ and did not prevent the inhibition elicited by ACEA. The ability of ACEA and AEA to reduce depolarization-evoked Ca^{2+} transients was significantly reduced in oligodendrocytes from CB_1 receptor knockout mice. Bath application of the inwardly rectifying K^+ channels (Kir channels) blockers $BaCl_2$ and $CsCl_2$ reduced the size of voltage-induced Ca^{2+} influx and partially prevented the inhibitory effect of ACEA. These results indicate that cannabinoids inhibit depolarization-evoked Ca^{2+} transients in oligodendrocytes via CB_1 receptor-independent and -dependent mechanisms that involve the activation of PTX-sensitive $G_{i/o}$ proteins and the blockade of Kir channels (Mato et al. 2009).

4. Characterization of new neuropeptide receptors

Many orphan receptors for which the ligands and function are still unknown are encoded by mammalian genomes. We focus on the characterization of a subset of these receptors expressed in specific brain regions, through the identification of their ligand and the delineation of their function. Cell lines coexpressing $G_{\alpha16}$ or $G_{\alpha qi5}$, apoaequorin and the selected orphan receptors (in its native form of fused to an epitope tag) have been established.

Two receptors were extensively screened using different types of brain extract and fractionation. In an extract made in a solution containing 90% methanol, 9% H_2O and 1% acetic acid, fractionated on a Poros column, no specific activities were detected in the aequorin-based assay. When the same type of extracts were made from hippocampus, and fractionated on a SAX column eluted with an NaCl gradient, a biological activity was detected for several (but not all) cell lines expressing one of the receptors. A milder signal was obtained from whole brain using the same protocol. We are presently testing the purification of this biological activity, as well as its specificity for the receptor of interest. In extracts dedicated to bioamines and other small molecules, no specific activities were detected either from total brain and from striatum.

A new knockout model for a brain receptor was obtained. Cohorts of mice were tested in behavioural settings. We have shown a mild motor impairment induced by the inactivation of the receptor gene. These modifications are relatively weak but constitute a set of characteristics of Parkinson disease and psychoaffective disorders. We will further investigate motor and affective disturbances in this model. We will also evaluate their sensitivity to neurotoxins.

5. Orphan receptors in other systems

The orphan Leucine-rich repeat G protein-coupled receptor 5 (LGR5/GPR49), a target of Wnt signaling, is a marker of adult intestinal stem cells (SC). However, neither its function in the adults, nor during development of the intestine have been addressed yet. We have investigated the role of LGR5 during ileal development by using LGR5 null/LacZ-NeoR knock-in mice. X-gal staining experiments showed that, after villus morphogenesis, Lgr5 expression becomes restricted to dividing cells clustered in the intervillus region and is more pronounced in the distal small intestine. At day E18.5, LGR5 deficiency leads to premature Paneth cell differentiation in the small intestine without detectable effects on differentiation

of other cell lineages, nor on epithelial cell proliferation or migration. Quantitative RT-PCR experiments showed that expression from the LGR5 promoter was upregulated in LGR5-null mice, pointing to the existence of an autoregulatory negative feedback loop in intact animals. This deregulation was associated with overexpression of Wnt target genes in the intervillus epithelium. Transcriptional profiling of mutant mice ileums revealed that LGR5 function is associated with expression of SC and SC niche markers. Together, our data identify LGR5 as a negative regulator of the Wnt pathway in the developing intestine (Garcia et al. 2009).

We have recently shown that Lgr4 knock-out (LGR4KO) male mice are infertile due to a developmental defect of the reproductive tract. Spermatozoa do not reach the epididymis and accumulate at the rete testis and efferent ducts. We have proposed that in LGR4KO, efferent duct might fail to connect resulting in blind-ended tubes that preclude the normal transit of sperm cells. To explore this possibility, we reconstructed the three-dimensional (3D) structure of the organ from serial microphotographs. The resulting model allowed to individualize and follow each efferent duct from the testis up to the epididymis, and to display the spatial distribution of their content. The transit of spermatozoa is indeed blocked in LGR4KO mice but, contrary to the expectation, the ducts connect normally to each other, forming a single tube that flows into the epididymis, as in the wild-type animals. In the KO however, transit of the sperm is abruptly blocked at the same level syncytial-like aggregates appear in the luminal space. The model also allowed calculating, for the first time, morphometric parameters of the mouse efferent duct, such as total volume, surface, radius, and length. These data unambiguously showed that efferent ducts in the mutant mouse are dramatically shortened and less convoluted than in the wild-type animal, providing an explanation to the phenotype observed in LGR4KO. Combined with in situ immunodetection or RNA in situ hybridization, 3D reconstruction of serial histological sections will provide an efficient mean to study expression profiles in organs which do not lend themselves to whole-mount studies (Lambot et al. 2009).

6. ChemR23

We identied previously chemerin as the natural ligand of the ChemR23 receptor. Chemerin is a chemoattractant factor for human immature dendritic cells (DCs), macrophages, and NK cells. We have now characterized chemerin/ChemR23 system in mouse, in terms of pharmacology, structure-function, distribution, and in vivo biological properties. Mouse chemerin is synthesized as an inactive precursor (prochemerin) requiring, as in human, the precise processing of its C-terminus for generating an agonist of ChemR23. Mouse ChemR23 is highly expressed in immature plasmacytoid DCs and at lower levels in myeloid DCs, macrophages, and NK cells. Mouse prochemerin is expressed in most epithelial cells acting as barriers for pathogens but not in leukocytes. Chemerin promotes calcium mobilization and chemotaxis on DCs and macrophages and these functional responses were abrogated in ChemR23 knockout mice. In a mouse model of acute lung inflammation induced by LPS, chemerin displayed potent anti-inflammatory properties, reducing neutrophil infiltration and inflammatory cytokine release in a ChemR23-dependent manner. ChemR23 knockout mice were unresponsive to chemerin and displayed an increased neutrophil infiltrate following LPS challenge. Altogether, the mouse chemerin/ChemR23 system is structurally and functionally conserved between human and mouse, and mouse can therefore be considered as a good model for studying the anti-inflammatory role of this system in the regulation of immune responses and inflammatory diseases (Luangsay et al. 2009).

In collaboration with an Italian group from Brescia, we have alsoi pursued the analysis of the chemerin/ChemR23 system in human inflammatory diseases of the skin and mucosa. Psoriasis is a type I interferon-driven T cell-mediated disease characterized by the recruitment of plasmacytoid dendritic cells (pDC) into the skin. The molecules involved in pDC accumulation in psoriasis lesions are unknown. Chemerin is the only inflammatory chemotactic factor that is directly active on human blood pDC in vitro. We evaluated therefore the role of the chemerin/ChemR23 axis in the recruitment of pDC in psoriasis skin. Prepsoriatic skin adjacent to active lesions and early lesions were characterized by a strong expression of chemerin in the dermis and by the presence of $CD15^+$ neutrophils and $CD123^+$/BDCA-2^+/$ChemR23^+$ pDC. Conversely, skin from chronic plaques showed low chemerin expression, segregation of neutrophils to epidermal microabscesses, and few pDC in the dermis. Chemerin expression was localized mainly in fibroblasts, mast cells, and endothelial cells. Fibroblasts cultured from skin of psoriatic lesions expressed higher levels of chemerin messenger RNA and protein than fibroblasts from uninvolved psoriatic skin or healthy donors and promoted pDC migration in vitro in a chemerin-dependent manner. Therefore, chemerin expression specifically marks the early phases of evolving skin psoriatic lesions and is temporally strictly associated with pDC. These results support a role for the chemerin/ChemR23 axis in the early phases of psoriasis development (Albanesi et al. 2009).

7. The FPRL2 receptor

Formyl peptide receptors (FPRs) are a small group of seven-transmembrane domain, G protein-coupled receptors that are expressed mainly by mammalian phagocytic leukocytes and are known to be important in host defense and inflammation. The three human FPRs (FPR1, FPR2/ALX, and FPR3) share significant sequence homology and are encoded by clustered genes. Collectively, these receptors bind an extraordinarily numerous and structurally diverse group of agonistic ligands, including N-formyl and nonformyl peptides of different composition, that chemoattract and activate phagocytes. N-formyl peptides, which are encoded in nature only by bacterial and mitochondrial genes and result from obligatory initiation of bacterial and mitochondrial protein synthesis with N-formylmethionine, is the only ligand class common to all three human receptors. Structural and functional studies of the FPRs have produced important information for understanding the general pharmacological principles governing all leukocyte chemoattractant receptors. We provided an overview of the discovery and pharmacological characterization of FPRs, to introduce an International Union of Basic and Clinical Pharmacology (IUPHAR)-recommended nomenclature, and to discuss unmet challenges, including the mechanisms used by these receptors to bind diverse ligands and mediate different biological functions (Ye et al. 2009).

We have pursued the study of FPRL2/FPR3, fot which a natural ligand (the peptide F2L) was discovered in the laboratory a few years ago. We investigated the detailed functional distribution of FPRL2 in leukocytes by quantitative PCR, flow cytometry, immunohistochemistry, and chemotaxis assays, with the aim of raising hypotheses regarding its potential functions in the human body. We described that FPRL2 is highly expressed and functional in plasmacytoid dendritic cells and up-regulated upon their maturation. FPRL2 is also expressed in eosinophils, which are recruited but do not degranulate in response to F2L. FPRL2 is expressed and functional in macrophages differentiated from monocytes in vitro in different conditions. However, in vivo, only specific subsets of macrophages express the receptor, particularly in the lung, colon, and skin, three organs chronically exposed to pathogens and exogenous aggressions. This distribution and the demonstration of the production of the F2L peptide

in mice underline the potential role of FPRL2 in innate immunity and possibly in immune regulation and allergic diseases (Devosse et al. 2009).

8. Dimerization of G protein coupled receptors

We have pursued the analysis of the functional consequences of the homo- and hetero-dimerization of GPCRs, using chemokine receptors as models. Using a combination of luminescence complementation and bioluminescence resonance energy transfer assays, we have demonstrated for the first time the existence of hetero-oligomeric complexes composed of at least three chemokine receptors (CCR2, CCR5, and CXCR4). We showed in T cells and monocytes that negative binding cooperativity takes place between the binding pockets of these receptors, demonstrating their functional interaction in leukocytes. We also showed that specific antagonists of one receptor (TAK-779 or AMD3100) lead to functional cross-inhibition of the others. Finally, using the air pouch model in mice, we showed that the CCR2 and CCR5 antagonist TAK-779 inhibits cell recruitment promoted by the CXCR4 agonist SDF-1□/CXCL12, demonstrating that cross-inhibition by antagonists also occurs in vivo. Thus, antagonists of the therapeutically important chemokine receptors regulate the functional properties of other receptors to which they do not bind directly with important implications for the use of these agents in vivo (Sohy et al. 2009).

9. Ligand processing and regulation of biological activity

The activity of many GPCR ligands, including chemokines, is regulated at the post-translational level by proteolysis or other types of modifications. We have studied in collaboration with other groups the post-translational modifications of several chemokines affecting their activity on the cognate receptors.

The CC chemokine CCL14a is constitutively expressed in a large variety of tissues and its inactive proform CCL14a(1-74) circulates in high concentrations in plasma. CCL14a(1-74) is converted into CCL14a(9-74) by the proteases urokinase-type plasminogen activator and plasmin and is a highly active agonist for the chemokine receptors CCR1 and CCR5. We have isolated a new CCL14a analog, CCL14a(12-74), from blood filtrate. To elucidate the functional role of the N terminus, a panel of N-terminally truncated CCL14a analogs were tested on the receptors CCR1 to CCR5 and on the human cytomegalovirus (HCMV)-encoded chemokine receptor US28. The rank order of binding affinity to these receptors and of the activation of CCR1 and CCR5-mediated intracellular Ca^{2+} concentration mobilization is CCL14a(6-74)<(7-74)<(8-74)<<(9-74) = (10-74)>>(11-74)>>(12-74). The almost identical affinities of CCL14a(7-74), CCL14a(9-74), and CCL14a(10-74) for the US28 receptor and the inhibition of US28-mediated HIV infection of 293T cells by all of the N-terminally truncated CCL14a analogs support the promiscuous nature of the viral chemokine receptor US28. In high concentrations, CCL14a(12-74) did reveal antagonistic activity on intracellular Ca^{2+} concentration mobilization in CCR1- and CCR5-transfected cells, which suggests that truncation of Tyr^{11} might be of significance for an efficient inactivation of CCL14a. A putative inactivation pathway of CCL14a(9-74) to CCL14a(12-74) may involve the dipeptidase CD26/dipeptidyl peptidase IV (DPPIV), which generates CCL14a(11-74), and the metalloprotease aminopeptidase N (CD13), which displays the capacity to generate CCL14a(12-74) from CCL14a(11-74). Our results suggest that the activity of CCL14a might be regulated by stringent proteolytic activation and inactivation steps (Richter et al. 2009)

We have also analyzed the modification of the CXC chemokine SDF1α/CXCL12 by peptidylarginine deiminase (PAD) that converts arginine residues into citrulline (Cit), thereby reducing the number of positive charges. The three NH_2-terminal arginines of CXCL12, Arg^8, Arg^{12}, and Arg^{20}, were citrullinated upon incubation with PAD. The physiologic relevance of citrullination was demonstrated by showing coexpression of CXCL12 and PAD in Crohn's disease. Three CXCL12 isoforms were synthesized for biologic characterization: CXCL12-1Cit, CXCL12-3Cit, and CXCL12-5Cit, in which Arg^8, Arg^8/Arg^{12}/Arg^{20}, or all five arginines were citrullinated, respectively. Replacement of only Arg^8 caused already impaired (30-fold reduction) CXCR4 binding and signaling (calcium mobilization, phosphorylation of ERK and protein kinase B) properties. Interaction with CXCR4 was completely abolished for CXCL12-3Cit and CXCL12-5Cit. However, the CXCR7-binding capacities of CXCL12-1Cit and CXCL12-3Cit were, respectively, intact and reduced, whereas CXCL12-5Cit failed to bind CXCR7. In chemotaxis assays with lymphocytes and monocytes, CXCL12-3Cit and CXCL12-5Cit were completely devoid of activity, whereas CXCL12-1Cit, albeit at higher concentrations than CXCL12, induced migration. The antiviral potency of CXCL12-1Cit was reduced compared with CXCL12 and CXCL12-3Cit and CXCL12-5Cit (maximal dose 200 nM) could not inhibit infection of lymphocytic MT-4 cells with the HIV-1 strains NL4.3 and HE. In conclusion, modification of CXCL12 by one citrulline severely impaired the CXCR4-mediated biologic effects of this chemokine and maximally citrullinated CXCL12 was inactive. Therefore, PAD is a potent physiologic down-regulator of CXCL12 function (Struyf et al. 2009).

10. References

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Role of protein synthesis in late long-term potentiation (L-LTP)

General background

Nowadays, neuroscientists agree on the fact that memories are stored in the brain as changes in the strength of synaptic connections between the neurons. In other words synaptic plasticity underlies memory.

In the CA1 region of hippocampal slices, stimulation of Schaffer collaterals by delivering one or several train of high-frequency stimulation induced an immediate and prolonged increase in synaptic strength called long-term potentiation (LTP). A single train triggers an early LTP (E-LTP) that lasts less than 3 h and depends only on modification and translocation of preexisting proteins. Several trains (usually 3 or 4) cause a long-lasting LTP (L-LTP) that persists more than 4 h. In contrast to E-LTP, L-LTP requires synthesis of new proteins. Like E-LTP, L-LTP is input-selective, that is, it is restricted to the activated synapses. Therefore, the newly synthesized proteins must be dispatched only to the activated synapses. According to Frey and Morris'model, this is achieved thanks to the creation in each stimulated synapse of a "tag" capable of capturing the products of the gene expression after their transport along dendrites. Assuming that a single train of stimulation – which is incapable of triggering transcription on its own – is able to create a synaptic tag, Frey and Morris's theory makes it possible to understand that, when L-LTP has been induced by a three or four-train stimulation in one pathway, the long-lasting aspect of that L-LTP can be captured by another pathway submitted only to a single train – a stimulation which would normally induce only a short-lasting LTP.

Two questions rise. (1) How does the "synaptic capture" work. (2) What is the nature of the newly synthesized proteins.

Experiments related to synaptic capture

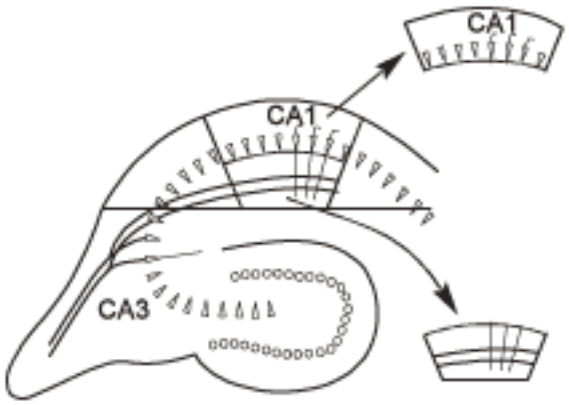
1. The synaptic-capture theory predicts that the development of the late phase of the LTP mediated by synaptic capture should be strongly dependent on protein synthesis. Surprisingly, anisomycin, an inhibitor of translation, was reported to inhibit this phenomenon only moderately. In our laboratory, we found that anisomycin was more effective in inhibiting the late phase of the L-LTP triggered by four trains when applied during the whole experiment rather than only around LTP induction, as is often done. Taking advantage of this observation, we have shown that the synaptic-capture mediated L-LTP is, as predicted by Frey and Morris'theory, strongly dependent on protein synthesis (NeuroReport 2009, **20**, 1572-1576).

2. In L-LTP, synaptic activation induces (1) a transcription followed by translocation of mRNA into the dendrites where they are eventually translated and (2) a translation of preexisting mRNAs. We were able to evoke an L-LTP in dendrites separated from their somas. We found that the late phase of L-LTP elicited in isolated CA1 dendrites could not be transferred by synaptic capture. This strongly suggests that the proteins resulting from translation of mRNAs in dendrites remain stuck on the synaptic site where they were synthesized (NeuroReport 2010).

Experiments related to the identification of newly synthesized proteins

The basic idea was to compare the relative quantities of several hundreds of proteins from samples originating from hippocampal slices where an L-LTP had been triggered pharmacologically by forskolin and IBMX, two agents inducing an increase in the concentration of cyclic AMP, on the one hand and from control hippocampal slices on the other hand.

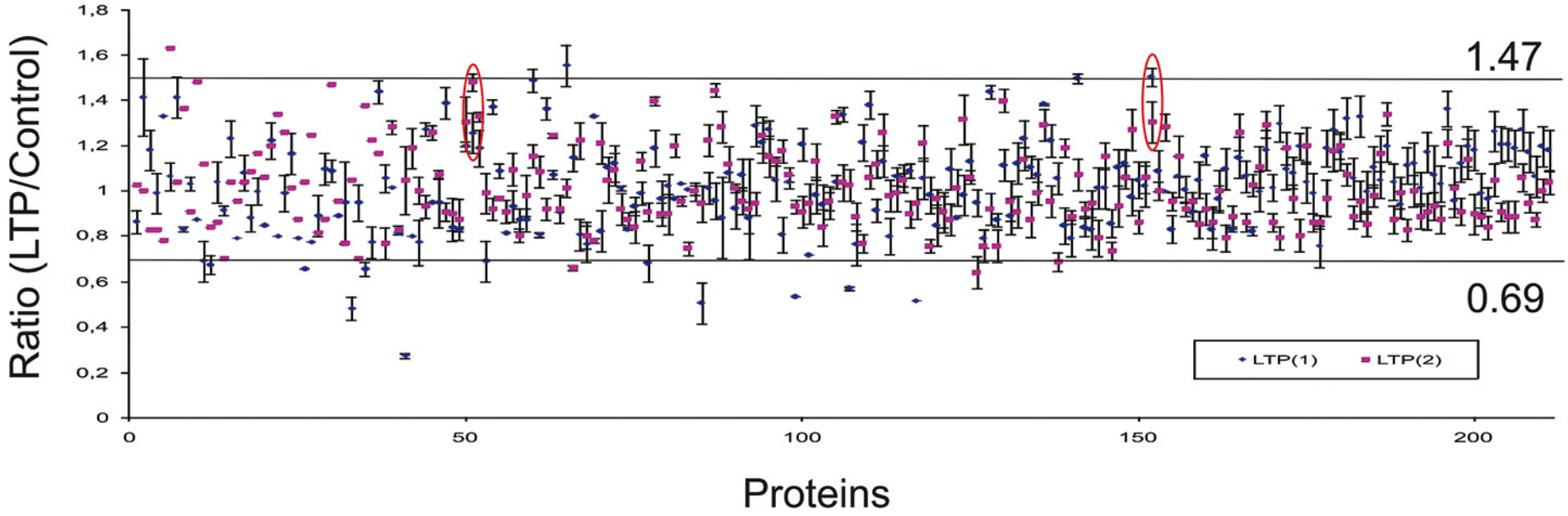
The proteins of the synaptoneurosomes from the CA1 dendritic region and the proteins from the CA1 somatic region were analysed 4 h after LTP induction.



The basic principle of the technique is a differential stable isotopic labeling (C^{12} and C^{13}) associated with a proteomics technique, the two-dimensional high pressure liquid chromatography (HPLC) coupled with electrospray tandem mass spectrometry (2D-LC/MS-MS). In the quantitative 2D-LC/MS-MS technique, the proteins extracted from either sample (control or treated) are coupled with succinimide which reacts with the terminal NH2 and the NH2 of the lysine residues. Succinimide is labeled with C^{13} for one sample and is natural (C^{12}) for the other. The two labeled protein pools are then mixed. The mixture obtained is submitted to trypsin and endoglu digestion. The resulting peptides are then separated by two-dimensional high pressure liquid chromatography and, after electrospray ionization, by mass spectrometry (MS). A same lysine-containing peptide will give birth to a pair of ionized peptides, the one labeled with C^{13} being slightly heavier than the other labeled with C^{12}. The ratio of the amounts of the two versions of the peptide gives the ratio of the amounts of the mother-proteins in the two hippocampal samples. Moreover, immediately after their separation, the peptides are fragmented by collision-induced dissociation. These fragments are then separated by MS (tandem mass spectrometry, MS-MS). This procedure allows “immediate” sequencing of the peptides and hence the identification of the mother-protein: the resultant spectral data are indeed automatically processed to search a genome sequence database for protein identification.

1. Synaptoneurosomal proteins

Two biological replicates were performed, each of them treated technically twice. Two hundred and twelve proteins were detected in both biological replicates. As shown in the figure below, the relative amount of most of them was found surprisingly unchanged. Only two proteins were significantly increased after LTP. They were Tau (Microtubule – associated protein) and SERCA (Sarcoplasmic reticulum Ca^{++} ATPase).


Ratio (LTP/Control)
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
50
100
150
200
Proteins
1.47
0.69
LTP(1)
LTP(2)

It is surprising that proteins described as overexpressed in LTP did not show any significant increase in our experiment.

	Sort of LTP	Ratio
eIF1A	electrical	1.03 ± 0.04
CaMKIIα	electrical	1.06 ± 0.15
AMPA1	electrical	1.06 ± 0.08

2. Proteins from the soma

Until now, only one biological essay was performed. The expression of 18 proteins was changed. The majority of them are linked to stress and to neurite growth.

Proteines	Accession	# (LTP/Ctrl)	Ratio (LTP/Ctrl)	SD	Fonctions
40S ribosomal protein S16	RS16_MOUSE	2	0.54	0.08	traduction
DnaJ homolog subfamily A member 2 (mDj3)	DNJA2_MOUSE	1	0.57	0	chaperone
Microtubule-associated protein tau	TAU_MOUSE	2	0.61	0.03	stabilisation microtubules
cAMP-dependent protein kinase type II-beta regulatory subunit	KAP3_MOUSE	2	0.62	0.02	activation fact. transcription
Dynamin-like 120 kDa protein, mitochondrial precursor (Large GTP-binding protein) (LargeG)	OPA1_MOUSE	2	0.63	0.007	apoptosis
Protein disulfide-isomerase A3 precursor (EC 5.3.4.1)	PDIA3_MOUSE	1	0.64	0	apoptosis
Mitochondrial precursor proteins import receptor	TOM70_MOUSE	1	0.64	0	importation protéines mitochondriales
Heat shock 70 kDa protein 4L	HS74L_MOUSE	1	0.65	0	chaperone
Succinate dehydrogenase [ubiquinone] iron-sulfur protein, mitochondrial precursor (EC 1.3.5.1)	DHSB_MOUSE	2	0.66	0.009	cycle de Krebs
Microtubule-associated protein 1B (MAP 1B)	MAP1B_MOUSE	6	0.66	0.09	stabilisation microtubules
Voltage-gated potassium channel subunit beta-2 (K(+) channel subunit beta-2)	KCAB2_MOUSE	1	0.67	0	transport ions
40S ribosomal protein S3a	RS3A_MOUSE	1	1.49	0	traduction
AP-1 complex subunit gamma-1	AP1G1_MOUSE	2	1.49	0.07	transport de protéines
Synaptic vesicle glycoprotein 2A	SV2A_MOUSE	2	1.53	0.005	transport de protéines
4F2 cell-surface antigen heavy chain	4F2_MOUSE	1	1.60	0	transport d'acides aminés
Vesicle-associated membrane protein 1 (VAMP-1) (Synaptobrevin-1)	VAMP1_MOUSE	1	1.64	0	transport de protéines
Peroxiredoxin-1 (EC 1.11.1.15)	PRDX1_MOUSE	1	1.91	0	Elimination des peroxides
Oligodendrocyte-myelin glycoprotein precursor	OMGP_MOUSE	2	2.05	0.005	myelination

Progress report of the research group of

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Roles of Specific Neuronal Populations in Functions and Disorders of Basal Ganglia

The basal ganglia system constitutes with the cerebral cortex an interconnected neural network involved in adaptive control of behaviour. The basal ganglia have a tremendous importance in human diseases as they are centrally affected in Parkinson's disease, Huntington's disease, schizophrenia or drug addiction. The striatum, the major input structure of this system is made up several neuronal populations including two efferent medium-size spiny neurons (MSN) sub-populations characterised by their outputs, either substantia nigra *pars reticulata* or globus pallidus (GP); as well as four classes of interneurons. The two populations of MSN, striatonigral and striatopallidal neurons, expressing dopamine D_1 (D_1R) or D_2 (D_2R) receptors, respectively, give rise to the direct and indirect pathways of the basal ganglia circuitry, respectively.

The major aims of our research group is to understand the differential functional properties of the two MSN populations, to determine the distinct roles of these neurons and hence of direct and indirect pathways as well as those of specific striatal interneuron sub-classes in basal ganglia physiology and their distinct involvement in basal ganglia pathologies, through the development of animal models bearing cellular or molecular 'lesions' by conditional transgenesis.

The main achievements obtained on the past year thanks to the support from FMRE/GSKE are summarized below.

1. Elucidation of the striatopallidal neuron's functions by gene targeting

To study of the specific roles of striatopallidal and striatonigral neurons, our first aim was to generate conditional transgenic mice. We have previously generated 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 (A_{2A}r-Cre) (de Kerchove d'Exaerde et al., 2006). In order to demonstrate the specificity of Cre expression in the striatopallidal neurons, these mice have been crossed with reporter strains expressing either β-galactosidase (Rosa26-LacZ) (Soriano 1999) or an analogue of GFP (Z/EG mice) (Novak et al., 2000). Co-localisation experiments using anti-enkephalin (Enk) antibody and LacZ activity detection showed that >80% of the LacZ neurons are also Enk+. The expression of eGFP was shown to be restricted in a subpopulation of striatal neurons which were never identified as striatonigral by retrograde labelling and who co-expressed D_2 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.

This final characterization of this striatopallidal neuron-specific Cre strain was part of the results published in Durieux et al., 2009.

1.a conditional and selective ablation of striatopallidal neurons

As a first attempt to gain insight on the roles of striatopallidal neurons and thanks to the generation of this specific A_{2A}r-Cre mice strain, we used the strategy of conditional and selective ablation of striatopallidal neurons by crossing these mice with mice allowing the conditional expression of the

diphtheria toxin receptor (rosa26-lox-stop-lox-DTR mice). Through the stereotaxic injection of diphtheria toxin, this allowed the specific ablation of striatopallidal neurons in the full striatum or selectively in sub-sectors of the striatum as the accumbens nucleus (Durieux et al., 2009). Since D_1R–striatonigral and D_2R–striatopallidal neurons are intermingled and morphologically indistinguishable they could not be functionally dissociated with classical techniques as chemical lesions or surgery. Therefore our mice strain was the first genetic model allowing selective ablation of these striatopallidal cells. We first demonstrated the very high selectivity and high efficacy of the ablation since neither markers (i.e. substance P or D_1R) of striatonigral neurons was decreased nor the density of the four classes of interneurons were affected whilst the specific markers (i.e. enkephalin, A_{2A}r or D_2R) of striatopallidal neurons were decreased by more than 90%. Importantly, by using Tyrosine Hydroxylase mRNA in situ hybridization, Dopamine Transporter binding and in vivo microdialysis, we found neither modifications in cell body or terminal dopaminergic markers nor differences in basal and amphetamine–induced dopamine overflow, indicating therefore that D_2R–striatopallidal neuron ablation does not induce major modifications in striatal dopaminergic function. As a first result demonstrating an alteration in the basal ganglia network consecutive to this ablation, we found an increase in GAD67 mRNA in the GP confirming that the D_2R–striatopallidal neurons exert an inhibitory control on GP GABA neuron activity (Durieux et al., 2009).

By using this strategy and examining the spontaneous locomotor activity of mice bearing a striatopallidal neuron loss in the entire striatum bilaterally, we showed that such ablation induced a marked (about 400%) and persistent (up to 40 days after injections) hyperlocomotion detected in open field video tracking (Durieux et al., 2009). It is worth to mention that this locomotor hyperactivity did not notably perturb food and water intakes as well as the circadian day-night cycle.

Since the ventral striatum is the key neuronal substrate for drug reinforcement and the roles of striatopallidal neurons in this area were completely unknown, we realized a restricted ablation of striatopallidal neurons in the nucleus accumbens. Surprisingly and unexpectedly, such ablation increased the preference for amphetamine in a conditioned place preference paradigm designed to model drug reward and reinforcement in rodents (Durieux et al., 2009). Moreover, in this condition, this preference was even maintained much longer (Durieux et al., 2009).

Altogether, our results provide direct experimental evidence that D_2R–striatopallidal neurons are critical for both the control of motor behaviour and drug reinforcement. They validated the hypothesis that these neurons exert inhibitory effect on the motor activity and demonstrated an unexpected involvement of these striatopallidal neurons in limiting the drug-reinforcement and motivational processes (Durieux et al., 2009).

1.b Specific inactivation of NR1 and cdk5 in striatopallidal neurons

The NMDA receptors seem to play a key role in reward and drug addiction (ventral striatum) and in motor learning (caudate-putamen). 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 to understand the specific roles of this receptor in each striatal subpopulation by the selective inactivation of this gene in these populations of neurons. The NR1 floxed ($NR1^{f/f}$) mice allowing a conditional inactivation of NR1 by the Cre recombinase have been double-crossed with our “striatopallidal-Cre” - A_{2A}R-Cre mice to obtain homozygous mice. The characterization of A_{2A}R-Cre/+ $NR1^{f/f}$ mice showed a selective decrease in NMDA receptor binding in the caudate-putamen and

accumbens nucleus as compared to the cerebral cortex. A first series of behavioural testing showed that the mice deficient in NMDA receptors in the striatopallidal neurons exhibit a hyperlocomotor activity similar to the one observed in mice without striatopallidal neurons (see above point 1.a). Moreover, evaluation in their ability to learn specific motor tasks by using two different tests showed that they exhibited a clear deficit in motor learning. Further analysis are required to evaluate the roles of these receptors in striatopallidal neurons in drug reinforcement. On the other hand, electrophysiological characterization of the glutamatergic cortico-striatal synaptic transmission in brain slices from these mice is running.

Cdk5 is a kinase that is involved in intracellular signaling cascades leading to neuroadaptation and synaptic plasticity and in drug addiction. Since its role in specific striatal populations and its involvement in the normal motor learning process or in striatal neuroadaptation in motor diseases are undetermined, we have similarly produced A_{2A}R-Cre/+ cdk5$^{f/f}$ mice in order to specifically inactivate this gene in striatopallidal neurons. We have obtained such mice and demonstrated that the cdk5 locus recombination occurs in our mouse strain. A first series of behavioural testing has been very recently realized and needs further validation.

1.c Gene profiling of striatonigral and striatopallidal neurons and characterization of striatopallidal neuron-specific genes

The striatopallidal-GFP mice (see point 1.a) have 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 was to establish the gene expression profiles of these different populations by microarrays. RNA amplification and identification was performed to validate the differential expression of some known genes (i.e. enkephalin, substance P, A_{2A} receptor) showing that we have an enrichment of more than 90%. We have identified a series of genes, which showed high differential expression in the striatopallidal neurons with ratio up to 100:1. This differential gene expression has been validated by using different techniques. Among these genes, we have selected genes as RGS5, IGFBP7, GuaCy13A, Adk that exhibit both a high differential expression and a putative physiological relevance for further analysis using different knock down strategies. One of these, RGS5, is a member of the large “Regulator of G Protein Signalling” family. It negatively regulates some G proteins and inhibit the signalling of Gαi, Gαq and Gαo and has been shown to be striatally up-regulated by an acute amphetamine treatment. We have started the behavioural study of full RGS5 knock-out that we obtained through collaboration. Up to now, we did not find any alteration in spontaneous locomotor activity, motor learning, drug sensitization or drug reinforcement processes.

1.d conditional targeting of nitrergic neurons

Besides the two populations of striatal efferent neurons (MSN) are four classes of interneurons. Among theses, the nitrergic, synthesizing NO, neurons have been suggested to play important roles. To gain insight to these functions, we had the objective to selectively ablated these neurons from the striatum and, hence, have started the construction of line of mice expressing the Cre recombinase under the control of the neuronal NO synthetase (nNOS) promoter (nNOS-Cre) by using the BAC strategy. Very succinctly, following steps of this construction have been realized: cloning of the Cre-recombinase in pbsk vector, cloning of the Cre-recombinase-3XSTOP-frt-Néo-frt construct in the BAC RP24-164C18, micro-injection of the recombined BAC. Five founders have been obtained and have been crossed with reporter mice Rosa26-LacZ in order to identify the sites of recombination events. The neuroanatomical characterization of these five lines of mice is in progress.

2. Regulation of striatal neurons excitability and corticostriatal synaptic transmission

Striatal neurons (MSN) excitability and corticostriatal synaptic transmission are tightly regulated both by a large series of neurotransmitters among which dopamine plays central role in learning rules and adenosine is a major neuromodulator, as well as through specific intrinsic somato-dendritic conductances that shape their responses. We previously studied the modulation of the glutamatergic corticostriatal transmission and plasticity by A_{2A} receptor (D'Alcantara et al., 2001 and reviewed in Schiffmann et al., 2007; Ferré et al., 2008).

We have now studied the mechanisms of the modulation exerted by D_2 and A_{2A} receptors on the MSN excitability (Azdad et al., 2009a). By using the perforated patch configuration of the patch clamp technique in combination with peptide occlusion protocols, we showed that dopamine D_2 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 specific subtype of calcium channels (CaV1.3). This D_2R-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., 2009a). Such effect was shown to be specific of the striatopallidal neuron population since it was only detected on GFP-expressing neurons in D_2R-GFP mice. This suggested that the action of A_{2A} receptor activation was completely or partially due to an intramembrane interaction such as D_2-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}-D_2 heteromerization. We showed that these peptides fully blocked the ability of A_{2A} receptor activation to counteract the D_2 effect demonstrating the involvement of D_2-A_{2A} heteromerization in this modulation. Not only our results demonstrated a strong D_2R-A_{2A}R antagonistic regulation of MSN excitability but they also supported for the first time in a physiological condition, the functional relevance of this heteromerization (Azdad et al., 2009a). In the same line, we have also showed that MSN intrinsic excitability is antagonistically regulated by D_2 and A_{2A} receptors through an additional mechanism involving the modulation of an inactivating A-type potassium current, I_A (Azdad and SNS unpublished results).

Dopamine has been previously shown to exert a variety of electrophysiological effects in MSN including the modulation of intrinsic conductances (i.e. see above) and the involvement in different types of corticostriatal synaptic plasticity. However, despite series of available data the effects resulting from dopamine depletion on the MSN intrinsic excitability remained incompletely documented and puzzling. To gain insight on these effects, we studied the alterations in MSN excitability and corticostriatal synaptic transmission in hypodopaminergic conditions mimicking Parkinson's disease (Azdad et al., 2009b). We showed, by performing perforated patch clamp recordings on brain slices, that dopamine depletion leads to an increase in MSN intrinsic excitability through the decrease of an inactivating A-type potassium current, I_A. Despite the large decrease in their excitatory synaptic inputs determined by the decreased dendritic spines density and the increase in minimal current to evoke the first EPSP, this increase in intrinsic excitability resulted in an enhanced responsiveness to their remaining synapses, allowing them to fire similarly or more efficiently following input stimulation than in control condition (Azdad et al., 2009b). Therefore, this increase in intrinsic excitability through the regulation of I_A represents a form of homeostatic plasticity allowing neurons to compensate for perturbations in synaptic transmission and to promote stability in firing. Such homeostatic plasticity has been demonstrated in a variety of physiological conditions such as memory storage or activity-dependent development in order to

adjust synaptic strengths and/or intrinsic excitability to promote stability but its existence and nature in pathological conditions were mostly unknown. Our observations (Azdad et al., 2009b) showed that this homeostatic ability to maintain firing rates within functional range also occurs in pathological conditions, allowing stabilizing neural computation within affected neuronal networks.

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Molecular genetics and biology of Charcot-Marie-Tooth neuropathies

1. Research report

Charcot-Marie-Tooth (CMT) disease is the most common inherited neuromuscular disorder occurring worldwide with a prevalence of 1/2500. CMT neuropathies are caused by a length-dependent degeneration of the peripheral nerves resulting in progressive weakness and wasting of foot and hand muscles, and distal sensory loss. Sometimes patients need walking aids or become wheelchair dependent even at a young age. Autosomal dominant, autosomal recessive and X-linked modes of inheritance have been described in CMT. Currently more than 40 disease associated genes and over 800 different pathogenic mutations have been identified for CMT and related inherited peripheral neuropathies. Different CMT phenotypes can be caused by mutations in the same gene, and conversely mutations in different genes may result in the same phenotype (i.e. genetic and phenotypic heterogeneity). This is further complicated by the fact that some mutations are private and occur in specific CMT subtypes or distinct ethnic groups. Mutations in more than 20 genes cause primary alterations of the myelin sheath (demyelinating phenotypes). Well-known examples are *myelin protein zero* (*MPZ*), *peripheral myelin protein 22* (*PMP22*) and *connexin 32* (*GJB1*). Mutations in genes expressed specifically in the axon however (e.g. *neurofilament light chain, NFL*), result in axonal CMT phenotypes. Their gene products have cell type specific functions and therefore the underlying disease pathomechanisms can be logically inferred. Other mutations have been reported to cause an intermediate CMT, with both demyelinating and axonal phenotypes. More recently, CMT causing mutations have been identified in ubiquitously expressed genes and the resulting proteins have housekeeping functions and pleiotropic activities in many different cells and tissues. Therefore, these genes were not obvious candidates for peripheral nerve degeneration and it remains an enigma why the mutant proteins cause such specific length-dependent degeneration of peripheral nerves.

The tremendous success of molecular genetic research performed in the past 15 years resulted from the availability of multiplex and/or consanguineous families suitable for linkage studies or homozygosity mapping. So far, most genes for CMT were identified through positional cloning or via a candidate gene approach. As large genetically unsolved CMT families have become rare, focus is now shifting towards smaller families and isolated patients. The availability of high-throughput genotyping platforms nowadays allows to tackle this still very large group of genetically unsolved patients. We have implemented innovative genetic and functional approaches to find novel disease causing genes, and to develop strategies to study the “not-obvious” CMT genes we recently identified. To find novel functional candidate genes, but also to identify peripheral nerve specific molecular pathways, we are now studying differential protein–protein interaction networks in CMT neuropathies. The identification of signaling cascades involved in neuro-degeneration and –protection will provide novel insights in regulatory pathways in heath and disease and will identify new candidate genes for peripheral neuropathies. Furthermore, we are developing mouse and fly models for selected CMT types. Altogether, our research will ultimately result in the identification of targets for therapeutic intervention, not only for one type of CMT, but hopefully for several CMT subtypes, including the more rare and/or complex phenotypes.

In this GSKE report 2009 we will first focus on our results recently obtained on the intermediate type of CMT neuropathies. Dominant Intermediate Charcot-Marie-Tooth (DI-CMT) neuropathy is a genetic and phenotypic variant of classical CMT characterized by intermediate nerve conduction velocities and histological evidence of both axonal and demyelinating features. So far specific mutations in *MPZ*, *GJB1* or *NEFL*, have been associated with DI-CMT. In addition, three loci were reported: DI-CMTA, DI-CMTB and DI-CMTC. Mutations in *dynamin 2* (*DNM2*) have been associated with DI-CMTB, and we recently identified mutations in *tyrosyl-tRNA synthetase* (*YARS*) in unrelated DI-CMTC families. YARS is the second aminoacyl-tRNA synthetase found to be involved in CMT, the other being *glycil-tRNA synthetase* (*GARS*) involved in CMT type 2D. Because mutations in *DNM2* and *YARS* have only recently been discovered, the mutation and clinical spectra of their defects as well as cellular pathomechanisms are still unknown and animal models are not yet available.

For DI-CMTB we studied the clinical, haematological, electrophysiological and sural nerve biopsy findings in 34 patients belonging to 6 unrelated families in whom a *DNM2* mutation was identified. Two *DNM2* mutations were novel, and in contrast to the other CMT-related mutations in *DNM2*, which are all located in the pleckstrin homology domain, these are in the middle domain and proline-rich domain of dynamin protein, respectively. We also reported the first disease-causing mutation in the proline-rich domain of dynamin. Patients with a *DNM2* mutation presented with a classical CMT phenotype, which was mild to moderately severe and only a few patients were wheelchair-bound. The mean age at onset was 16 years with a large variability ranging from 2 to 50 years. Interestingly, in two families which carry two different mutations affecting the same amino acid (Lys558), CMT cosegregated with neutropaenia (an abnormally low number of neutrophils). In addition, early onset cataracts were observed in one of the families. The electrophysiological data indicated intermediate or axonal motor median nerve conduction velocities (NCV) ranging from 26 m/s to normal values in four families, and less pronounced reduction of motor median NCV (41-46 m/s) with normal amplitudes in two families. Sural nerve biopsy in one patient showed diffuse loss of large myelinated fibres, presence of many clusters of regenerating myelinated axons and fibres with focal myelin thickenings. We concluded that *DNM2* mutations should be screened in the autosomal dominant CMT families with intermediate or axonal NCV, and in patients with a classical mild to moderately severe CMT phenotype, especially when CMT is associated with neutropaenia or cataracts (Claeys et al. 2009).

For DI-CMTC, we previously identified 3 dominant disease-causing mutations in the *YARS* gene, encoding tyrosyl-tRNA synthetase (TyrRS). We have now generated a *Drosophila* model of DI-CMTC. Expression of the three mutant –but not wild type– TyrRS in *Drosophila* recapitulates several hallmarks of the human disease, including a progressive deficit in motor performance, electrophysiological evidence of neuronal dysfunction and morphological signs of axonal degeneration. Not only ubiquitous, but also neuron-specific expression of mutant TyrRS, induces these phenotypes, indicating that the mutant enzyme has cell-autonomous effects in neurons. Furthermore, our biochemical and genetic complementation experiments revealed that loss of enzymatic activity is not a common feature of DI-CMTC associated mutations. Thus, we demonstrated that the DI-CMTC phenotype is not due to haploinsufficiency of aminoacylation activity, but most likely to a *gain-of-function* alteration of the mutant TyrRS or interference with an unknown function of the wild type protein. The dysfunction induced by both human and fly mutant TyrRS expression also suggests that the molecular pathways leading to mutant TyrRS associated neurodegeneration are conserved from flies to humans. We anticipate that our model will contribute to novel insights on the pathomechanisms of CMT and linking aminoacyl-tRNA synthetase mutations to nervous system degeneration (Storkebaum & Gonçalves et al. 2009). After having established the fly model where toxicity induced by DI-CMTC associated mutations can be

measured in a variety of assays, we are now interested in defining the molecular processes that lead to the observed dysfunction. To achieve this we are performing an unbiased modifier screen that will lead to the identification of genetic modulators of DI-CMTC associated phenotypes identified in our model. Here we are making use of publicly available collections of *Drosophila* mutants where single X-linked fly genes can be targeted. Our approach consists of a *gain-of-function screen* where mutant YARS is co-expressed with putative modifier genes in the fly retina (GMR-Gal4 driver) and *rough-eye enhancers* are identified. The genetic modifiers found in this screen will be further verified in our panel of behavioural assays in order to select the genes specifically modulating the neurodegenerative phenotypes and to reject the false-positive hits. The list of genetic interactors will be further investigated *in silico* and *in vivo.* By retrieving the available information concerning biological roles/relevance for nervous system function and after cross-validation in other CMT models, we will filter for the authenticity of our findings. Modifier genes identified in the fly model will be analyzed for genomic sequence variants in CMT patients and their segregation with the phenotype will be checked. In addition, RNA and protein expression levels of the modifying alleles will be determined using lymphoblast or fibroblasts cell lines of our CMT patients.

The second focus in this GSKE report 2009 is on our molecular genetic and biology results obtained for hereditary sensory and autonomic neuropathies (HSAN). These neuropathies are clinically and genetically heterogeneous disorders characterized by axonal atrophy and degeneration, exclusively or predominantly affecting the sensory and autonomic neurons. So far, disease-associated mutations have been identified in seven genes: two genes for autosomal dominant (*SPTLC1* and *RAB7*) and five genes for autosomal recessive forms of HSAN (*WNK1/HSN2, NTRK1, NGFB, CCT5* and *IKBKAP*). We performed a systematic mutation screening of the coding sequences of six of these genes on a cohort of 100 familial and isolated patients diagnosed with HSAN. In addition, we screened the functional candidate gene *NGFR (p75/NTR)* encoding the nerve growth factor receptor. We identified disease-causing mutations in *SPTLC1, RAB7, WNK1/HSN2* and *NTRK1* in 19 patients, of which three mutations have not previously been reported. The phenotypes associated with mutations in *NTRK1* and *WNK1/HSN2* typically consisted of congenital insensitivity to pain and anhidrosis (CIPA), and early-onset ulcero-mutilating sensory neuropathy, respectively. *RAB7* mutations were only found in patients with a Charcot-Marie-Tooth type 2B (CMT2B) phenotype, an axonal sensory-motor neuropathy with pronounced ulcero-mutilations. In *SPTLC1*, we detected a novel mutation in a previously unknown severe and early-onset HSAN phenotype. No mutations were found in *NGFB, CCT5* and *NGFR*. Overall disease-associated mutations were found in 19% of the studied patient group, suggesting that additional genes are associated with HSAN. Our genotype-phenotype correlation study broadens the spectrum of HSAN and provided additional insights for molecular and clinical diagnosis (Rotthier & Baets et al. 2009). In a separate manuscript we reported a young patient with CIPA, an autosomal recessive disorder caused by mutations in the *NTRK1* gene, which encodes the receptor for nerve growth factor. In addition to recurrent dislocation of the left hip joint and avascular necrosis of the left talus, the patient also presented with recurrent infections secondary to hypogammaglobulinemia, a feature not previously known to be associated with CIPA. The implication of the immune system of the reported patient broadens the clinical phenotype associated with NTRK1 mutations (Kilic et al. 2009).

We performed a biochemical study of *SPTLC1* mutations in collaboration with Dr. T. Hornemann (Zürich). Mutations in *SPTLC1* cause autosomal dominant hereditary sensory neuropathy type 1 (HSAN I). SPTLC1 is a subunit of the serine palmitoyltransferase (SPT). Four missense mutations in SPTLC1 were reported to cause HSAN I. The SPT catalyses the condensation of Serine and Palmitoyl-CoA, which is the first and rate-limiting step in the *de novo* synthesis of ceramides. Earlier studies showed that C133W and C133Y mutants have a reduced activity, whereas the impact of the V144D and G387A mutations

on the human enzyme was not tested yet. We show that none of the HSAN I mutations interferes with SPT complex formation. We demonstrate that also V144D has a reduced SPT activity, however to a lower extent than C133W and C133Y. In contrast, the G387A mutation showed no influence on SPT activity. Furthermore, the growth phenotype of an SPTLC1 deficient CHO cell line could be reversed by expressing either the wild-type SPTLC1 or the G387A mutant, but not the C133W mutant. This indicates that the G387A mutation is most likely not directly associated with HSAN I. Our findings were genetically confirmed by the identification of a nuclear HSAN family which showed segregation of the G387A variant as a non-synonymous single nucleotide polymorphism (Hornemann et al. 2009).

Finally, in collaboration with I. Kurth and C. Huebner (Jena) we identified a novel gene for HSAN type II. They showed that loss-of-function mutations in the *FAM134B* gene, encoding a newly identified cis-Golgi protein, cause HSAN II. The Fam134b knockdown resulted in structural alterations of the cis-Golgi compartment and induced apoptosis in primary dorsal root ganglion neurons. This implicates that FAM134B is a critical protein involved in long-term survival of nociceptive and autonomic ganglion neurons. For this study we screened 75 unrelated individuals with clinical symptoms compatible with HSAN and revealed homozygous loss-of-function *FAM134B* mutations in 3 families of our HSAN cohort. In all affected individuals disease onset was early, with impaired nociception complicated by ulcerations of hands and feet and chronic osteomyelitis leading to progressive acro-osteolysis with amputations. Nerve conduction velocities showed an exclusive or predominantly axonal sensory neuropathy (Kurth et al. 2009).

In conclusion, in 2009 we identified a novel disease causing gene for HSAN, once more a gene with an unknown function in the peripheral nervous system. We made the first *Drosophila* model for CMT neuropathy and more specifically for the DI-CMTC phenotype caused by mutations in the tyrosyl amino-acyl-tRNA synthetase YARS. We performed genotype-phenotype correlations in large cohorts of patients with sensory neuropathies and intermediate CMT.

2. Research Activities:

Articles in International Journals with acknowledgement to GSKE:

- Kurth,I., Pamminger,T., Hennings,C.J., Soehendra,D., Huebner,A.K., Rotthier,A., Baets,J., Senderek,J., Topaloglu,H., Farrell,S.A., Nürnberg,G., Nürnberg,P., De Jonghe,P., Gal,A., Käther,C., Timmerman,V., Hübner,C.A.: Mutations in FAM134B, encoding a newly identified Golgi protein, cause severe sensory and autonomic neuropathy. Nature Genetics 41(11): 1179-1181 (2009) (PMID: 19838196) (I.F.: 30.259)
- Rotthier,A.*, Baets,J.*, De Vriendt,E., Jacobs,A., Auer-Grumbach,M., Levy,N., Bonello-Palot,N., Kilic,S.S., Weis,J., Nascimento,A., Swinkels,M., Kruyt,M., Jordanova,A., De Jonghe,P., Timmerman,V.: Genes for hereditary sensory and autonomic neuropathies: a genotype-phenotype correlation. Brain 132(Pt 10): 2699-2711 (2009) (PMID: 19651702) (I.F.: 9.603), * equal contribution for the first authors.
- Claeys,K., Züchner,S., Kennerson,M., Berciano,J., García,A., Verhoeven,K., Storey,E., Merory,J., Bienfait,H.M.E., Lammens,M., Nelis,E., Baets,J., De Vriendt,E., Berneman,Z., De Veuster,I., Vance,J., Nicholson,G., Timmerman,V., De Jonghe,P.: Phenotypic spectrum of dynamin 2 mutations in Charcot-Marie-Tooth neuropathy. Brain 132(Pt 7): 1741-1752 (2009) (PMID: 19502294) (I.F.: 9.603)
- Storkebaum,E.*, Gonçalves,R.*, Godenschwege,T., Nangle,L., Mejia,M., Bosmans,I., Ooms,T., Jacobs,A., Van Dijck,P., Yang,X-L, Schimmel,P., Norga,K., Timmerman,V., Callaerts,P., Jordanova,A.: Dominant mutations in the tyrosyl-tRNA synthetase gene recapitulate in Drosophilafeatures of human Charcot-Marie-Tooth neuropathy. Proceedings of the National Academy of Sciences of the USA 106(28): 11782-11787 (2009) (PMID: 19561293) (I.F.: 9.38) * equal contribution for the first authors.

- Fabrizi,G.-M., Taioli,F., Ferrari,S., Bertolasi,L., Casarotto,M., Rizzuto,N., Deconinck,T, Timmerman,V., De Jonghe,P.: Further evidence that mutations in FRABIN/FGD4 cause Charcot-Marie-Tooth disease type 4H. Neurology 72(13): 1160-1164 (2009) (PMID: 19332693) (I.F.: 7.043)
- Hornemann,T., Penno,A., Richard,S., Nicholson,G., van Dijk,F.S., Rotthier,A., Timmerman,V., von Eckardstein,A.: A systematic comparison of all mutations in hereditary sensory neuropathy type I (HSAN I) reveals that the G387A mutation is not disease associated. Neurogenetics 10(2): 135-143 (2009) (PMID: 19132419) (I.F.: 3.0)
- Kilic,S.S., Ozturk,R., Sarisozen,B., Rotthier,A., Baets,J., Timmerman,V.: Humoral immunodeficiency in congenital insensitivity to pain with anhidrosis. Neurogenetics 10(2): 161-165 (2009) (PMID: 19089473) (I.F.: 3.0)
- Baets,J., Dierick,I., Ceuterick-de Groote,C., Van Den Ende,J., Martin,J-J., Geens,K., Robberecht,W., Nelis,E., Timmerman,V., De Jonghe,P.: Peripheral neuropathy and 46XY gonadal dysgenesis: a heterogeneous entity. Neuromuscular Disorders 19(2): 172-175 (2009) (PMID: 19167223) (I.F.: 2.932)
- Gallardo,E., García,A., Ramòn,C., Maraví,E., Infante,J., Gastón,I., Alonso,A., Combarros,O., De Jonghe,P., Berciano,J.: Charcot-Marie-Tooth disease type 2J with MPZ Thr124Met mutation: Clinico-electrophysiological and MRI study of a family. Journal of Neurology (2009) (PMID: 19629567)(Epub ahead) (I.F.: 2.536)

Other topic related articles in international journals:

- Haberlova,J., Claeys,K., De Jonghe,P., Seeman,P.: Cranial nerves palsy as an initial feature of an early onset distal hereditary motor neuropathy--a new distal hereditary motor neuropathy phenotype. Neuromuscular Disorders 19(6): 427-428 (2009) (PMID: 19409784) (I.F.: 2.932)

Scientific Prizes:

- **Jonathan Baets**: Pfizer Wetenschappelijke Prijs van de Faculteit Geneeskunde 2009: “Genes for hereditary sensory and autonomic neuropathies: a genotype-phenotype correlation”.

Awards and fellowships:

- **Annelies. Rotthier**: EMBO short term fellowship: “Functional characterization of mutations in SPTLC1 and SPTLC2 associated with hereditary sensory neuropathies” ASTF number: 441.2008 / 59.00-2009; Institute Clinical Chemistry, University Hospital Zürich, Switzerland; 2 December 2008 – 27 February 2009
- **Annelies. Rotthier**: Travel fellowship of the Peripheral Nerve Society (PNS) to attend the 2009 Biennial PNS Meeting, Wurzburg, Germany, July 4-8 (2009)

PhD theses:

- None in 2009

Students (masters and bachelors)

- **Ben De Clerck:** “Optimalisatie van de Proximity Ligation Assay (PLA) methode als uitleessysteem voor TLR activering” (Master stage Biochemistry)
- **Jascha Vervoort:** “Effect van DI-CMTC mutaties in *YARS* op celgroei en eiwitinteracties” (Master stage Biochemistry)
- **Veerle Smits:** “Expressie van Nod-like receptors in het perifere en het centrale zenuwstelsel” (Master thesis Biochemistry)
- **Yves Dondelinger:** “Zoektocht naar nieuwe pathomechanismen voor CMT-geassocieerde HSPB1 en HSPB8 mutanten” (Master thesis Biochemistry)
- **Josephine Roberts:** “Mutatieanalyse van PRX bij vroeg beginnende vormen van de ziekte van Charcot-Marie-Tooth” (Professional Bachelor thesis)

Contribution to international meetings:

- **Vincent Timmerman:** Peripheral Nerve Society Meeting: Chair Session 6 – Hereditary Neuropathies, Würzburg, Germany, July 4-8 (2009)
- **Vincent Timmerman** and **Peter De Jonghe:** Third International Charcot-Marie-Tooth Consortium Meeting: Organizers, Antwerp, Belgium, July 9-11 (2009)
- **Peter De Jonghe:** Third International Charcot-Marie-Tooth Consortium Meeting: Chair Session 5 – HSAN and new forms of neuropathies, Antwerp, Belgium, July 9-11 (2009)
- **Albena Jordanova:** Third International Charcot-Marie-Tooth Consortium Meeting: Chair Session 7 – Pathomechamisms of GDAP1, YARS and GARS mutations, Antwerp, Belgium, July 9-11 (2009)

– **Vincent Timmerman:** Third International Charcot-Marie-Tooth Consortium Meeting: Chair Session 8 – Immune mediated neuropathies and models for peripheral neuropathies, Antwerp, Belgium, July 9-11 (2009)

Invited Lectures at international meetings:

– **Vincent Timmerman**: Animal models for peripheral neuropathies. EU COST B30 meeting on animal models in research on neurodegeneration and neuroplasticity, Antwerp, Belgium, March 29-30 (2009)
– **Vincent Timmerman:** Unraveling the molecular genetics and biology of inherited peripheral neuropathies. International Symposium on Neuromuscular diseases, recent advances and translation to therapy, Madrid, Spain, May 28-29 (2009)
– **Albena Jordanova**: Unraveling the role of aminoacyl-tRNA synthetases in neurodegeneration: come fly with us. 2nd International Conference on Aminoacyl-tRNA-Synthetases: From the Genetic Code to Human Diseases and Medicine, San Diego, CA, USA, April 1-5 (2009)
– **Albena Jordanova:** Role of aminoacyl-tRNA synthetases in the peripheral nervous system: lessons from Drosophila. 11th International Congress on Amino Acids, Peptides and Proteins, Workshop: Aminoacyl-tRNA synthetase in signalling and disease, Vienna, Austria, August 3-7 (2009)

Invited Lectures at national meetings:

– **Albena Jordanova**: Unraveling the role of aminoacyl-tRNA synthetases in neurodegeneration: come fly with us. Seminar at the Max-Planck-Institute for Biophysical Chemistry, University of Göttingen, Germany, February 26-27 (2009)
– **Vincent Timmerman**: The molecular variability of inherited peripheral neuropathies, Deutschen Gesellshaft für Muskelkranke. German Society for Muscle Disease, Darmstadt, Germany, March 4-7 (2009)
– **Albena Jordanova**: Genetic studies in Gypsies – the Bulgarian experience. Seminar at the Department of Medical Genetics, VUB, Brussels, Belgium, April 2 (2009)
– **Sophie Janssens**: Possible involvement of TLRs in JNK expression upon acute neurodegeneration, University College London (Laboratory of Prof. K. Jessen and Prof. R. Mirsky), UK, August 6 (2009)
– **Albena Jordanova**: Genetische studies in de zigeunerpopulatie: ervaringen uit Bulgarije ideeën voor België. Integratiecentrum Foyer, Brussels, Belgium, September 9 (2009)
– **Peter De Jonghe** en **Annelies Rotthier**: Overzicht van het CMT onderzoek, Nationale Studie- en Contactdag van CMT België v.z.w, Antwerpen, November 14 (2009)

Slide presentations selected at international meetings:

– **Sophie Janssens**: TLR expression in the peripheral nerve. Journal of the Peripheral Nervous System 14 (Suppl 2): 148-149 (2009), 2009 Biennial PNS Meeting, Wurzburg, Germany, July 4-8 (2009)
– **Annelies Rotthier**: Mutation analysis of genes for hereditary sensory and autonomic neuropathies: Identification of new mutations and a genotype-phenotype correlation study. Journal of the Peripheral Nervous System 14 (Suppl 2): 128-129 (2009), 2009 Biennial PNS Meeting, Wurzburg, Germany, July 4-8 (2009)
– **Leonardo de Almeida Souza**: How do CMT-related mutations in HSPB1 affect its biochemical properties? Third International Charcot-Marie-Tooth Consortium Meeting, Antwerp, Belgium, July 9-11 (2009)
– **Ricardo Gonçalves**: Towards a better understanding of DI-CMTC, a contribution from Drosophila. Third International Charcot-Marie-Tooth Consortium Meeting, Antwerp, Belgium, July 9-11 (2009)

Poster presentations at international meetings

– **Ricardo Gonçalves:** From YARS mutations to a peripheral neuropathy, a flying perspective. 104th Annual Meeting of the Anatomische Gesellschaft, Antwerpen, Belgium, March (2009)
– **Jonathan Baets:** ARSACS in patients initially referred as CMT. Third International Charcot-Marie-Tooth Consortium Meeting, Antwerp, Belgium, July 9-11 : P29 (2009)
– **Jonathan Baets:** Genetic spectrum of hereditary peripheral neuropathies with onset in the first year of life. Third International Charcot-Marie-Tooth Consortium Meeting, Antwerp, Belgium, July 9-11 : P28 (2009)
– **Magdalena Zimon:** Large scale genetic approach for the molecular characterization of autosomal-recessive Charcot-Marie-Tooth disease. Third International Charcot-Marie-Tooth Consortium Meeting, Antwerp, Belgium, July 9-11 : P48 (2009)
– **Magdalena Zimon:** Novel mutations bring novel insight into GDAP1-associated CMT neuropathies. Third International Charcot-Marie-Tooth Consortium Meeting, Antwerp, Belgium, July 9-11 : P47 (2009)
– **Anne Holmgren:** Mutant HSPB8 and HSPB1 impairs formation of stable neurofilament network. Third International Charcot-Marie-Tooth Consortium Meeting, Antwerp, Belgium, July 9-11 : P40 (2009)

- **Sophie Janssens:** Induction of TLR expression in the peripheral nerve upon neurodegeneration. Third International Charcot-Marie-Tooth Consortium Meeting, Antwerp, Belgium, July 9-11 : P51 (2009)
- **Annelies Rotthier:** Mutation analysis of genes for hereditary sensory and autonomic neuropathies: Identification of new mutations and a genotype-phenotype correlation study. Third International Charcot-Marie-Tooth Consortium Meeting, Antwerp, Belgium, July 9-11: P37 (2009)
- **Ricardo Gonçalves:** Dominant mutations in the tyrosyl-tRNA synthetase gene recapitulate in Drosophila features of human Charcot-Marie-Tooth neuropathy. 21st European Drosophila Research Conference, Nice, France, November 18-21, P-102 (2009)

Slide presentations selected at national meetings:

- **Annelies Rotthier**: Mutation analysis of genes for hereditary sensory and autonomic neuropathies: Identification of new mutations and a genotype-phenotype correlation study. Annual Scientific IAP P6/43 meeting, Brussels, Belgium, October 26 (2009)
- **Ricardo Gonçalves**: Introducing flies to Charcot-Marie-Tooth disease, the YARS example. VIB Science Club Neurodegenerative Diseases II, Provinciehuis Leuven, Belgium, October 30 (2009)

Poster presentations at national meetings:

- **Annelies Rotthier**: Mutation analysis of genes for hereditary sensory and autonomic neuropathies: identification of new mutations and a genotype-phenotype correlation study. VIB Seminar 2009, Blankenberge, Belgium, March 12 : P92 (2009)
- **Sophie Janssens**: Induction of TLR expression in the peripheral nerve upon neurodegeneration. VIB Seminar 2009, Blankenberge, Belgium, March 12 : P72 (2009)
- **Joy Irobi:** Mutant heat shock protein HSPB8 induces aggregation and a pro-apoptotic phenotype in distal motor neuropathy. VIB Seminar 2009, Blankenberge, Belgium, March 12 (2009)
- **Joy Irobi:** Distal hereditary motor neuropathy caused by mutant HSPB8 reduced cell viability and induced protein aggregation. Third International Charcot-Marie-Tooth Consortium Meeting, Antwerp, Belgium, July 9-11 (2009)
- **Joy Irobi:** Mutant heat shock protein HSPB8 induces aggregation and a pro-apoptotic phenotype in distal motor neuropathy. Annual Scientific IAP P6/43 meeting, University of Brussels (ULB), Brussels, Belgium, October 26 (2009)
- **Jonathan Baets:** Genetic spectrum of hereditary peripheral neuropathies with onset in the first year of life. Annual Scientific IAP6/43 meeting, University of Brussels (ULB), Brussels, Belgium, October 26 (2009)
- **Magdalena Zimon**: Novel mutations in GDAP1 causing both dominant and recessive CMT disease. 9th annual meeting of the Belgian Society of Human Genetics: Darwin's 200th Birthday, ULB, Brussels, Belgium, February 13 (2009)
- **Magdalena Zimon:** Novel mutations bring novel insight into GDAP1-associated CMT neuropathies. Annual Scientific IAP6/43 meeting, University of Brussels (ULB), Brussels, Belgium, October 26 (2009)

Progress report of the research group of

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Progranulin in Neurodegenerative Dementia: Genetic, Functional and Neuropathological Characterization

Specific Aims of the Project

1. To evaluate the occurrence of complex *PGRN* null-mutations in a collection of 190 Belgian FTLD patients as well as study the contribution of *PGRN* mutations in FTLD-associated neurodegenerative diseases including PD, ALS and AD.

2. To identify genes modifying the highly variable onset age of FTDU associated with *PGRN* mutations.

3. To construct *Grn* knockout mice and illustrate whether $Grn^{-/+}$ and $Grn^{-/-}$ mice have neuronal loss especially in the basal forebrain or develop behavioral or cognitive abnormalities compared to the wild-type mice or to mice overexpressing human wild-type PGRN. However, because *PGRN* is an important gene expressed in a variety of tissues, the targeting construct utilizes a conditional knockout approach and will be utilized if the constitutive Grn loss is embryonically lethal. Moreover, this approach would also allow tissue- or cell type-specific *Grn* ablation.

4. To develop *PGRN* overexpressing and deficient cellular models such as primary neuronal cortical neurons derived from $Grn^{-/-}$ mice or RNAi-silenced immortalized neuron-like cells (SH-SY5Y, P19, and NTera) and non-neuronal cells (HEK293) and to utilize these models to study overexpressed and/or endogenous PGRN cellular localizations and protein trafficking and turnover. Moreover, these models will also be utilized to study PGRN-mediated cell proliferation or other phenotypic changes as well as altered cell-signaling pathways, inter alia, PI3K and MAPK pathways.

Progress Report

Specific aim 1 – *GRN* mutation spectrum

To characterize the diversity of mutation types that can lead to loss or reduction of PGRN function was a major aim of the project and has largely been addressed in the previous reporting period 2008. We identified small indels, splice site mutations and nonsense mutations associated with a premature protein translation termination codon and associated with nonsense-mediated mRNA degradation (NMD). We also identified for the first time a whole-gene deletion. Further, we identified missense mutations that were associated with reduced PGRN function, as measured by serum PGRN protein levels. Mutations were found to lead to different clinical manifestations of neurodegeneration, besides frontotemporal lobar degeneration, also including Alzheimer's disease and Parkinson's disease.

To evaluate serum PGRN levels as a biomarker for FTLD, we used an ELISA to measure in serum the PGRN protein levels of 6 affected and 8 unaffected carriers from within an extended Belgian founder FTLD-U family segregating the null mutation (IVS1 +5G>C). Further, we measured serum PGRN levels in 2 patients with other null mutations, in 4 patients carrying a predicted pathogenic missense mutation and in 5 patients carrying a benign missense polymorphism, in 9 unaffected non-carrier relatives and in 22 community control individuals. Serum PGRN levels were reduced in both affected and unaffected null mutation carriers compared to non-carriers (p exact <0.0001), and allowed perfect discrimination

between carriers and non-carriers (sensitivity: 1.0, 1-specificity: 0.0). Serum PGRN levels in Cys139Arg and Arg564Cys mutation carriers were significantly lower than in control individuals, but higher than in null mutation carriers, fitting the hypothesis of partial loss-of-function due to these missense mutations. As expected, levels for carriers of benign missense polymorphisms were not significantly different from control individuals. Our results indicate that the serum PGRN level is a reliable biomarker for diagnosis and early detection of FTLD-U caused by *GRN* null mutations, and provided the first *in vivo* evidence that at least some missense mutations in *GRN* may lead to a (partial) loss of PGRN (Sleegers *et al.*, 2009).

Where the focus of previous studies was on mutations affecting the coding region of the *GRN* gene, we also investigated the effect of noncoding, regulatory *GRN* variants. It has been claimed that homozygosity of the SNP rs5848 located in the 3'UTR of *GRN* increases risk for FTLD. The authors proposed that homozygosity of the T allele of rs5848 increases binding of the microRNA miR-659 which leads to an inhibition of *GRN* translation. However, the genetic association was only demonstrated in a single cohort. Given that association studies are fraught with problems of replication, we undertook the first replication of this data in three separate European FTLD cohorts representing a total of 467 patients and 1049 controls. No association with FTLD was observed in any individual cohort nor was any observed when the data was combined. Also, we did not identify significant association in the groups of FTLD patients with confirmed TDP43 neuropathology. These data argue that rs5848 is not a risk factor for FTLD (Rollinson *et al.* 2009).

Specific aim 2 – FTLD-GRN modifier genes

We performed a genome-wide linkage scan for genes modifying the variable onset age in the extended Belgian founder family DR8, segregating the *GRN* IVS1+5G>C null mutation. In preliminary studies, we excluded the apolipoprotein E gene (*APOE*) known to modify onset age in AD. Also, we found that serum levels of PGRN in all mutation carriers was about 50% of the levels observed in non-carriers, suggesting that the PGRN protein level produced from the unaffected gene copy is not a determinant of onset age 2. In the genome-wide STR-based linkage mapping study, in which we treated onset age as a censored quantitative trait we observed that one single quantitative trait locus (QTL) explained up to 91% of genetic variability corresponding to 65% of the total variability in onset age in this family. This QTL is mapped to a 7 Mb region and contains > 100 genes, none of which is associated with FTLD or another neurodegenerative disease.

Specific aim 3 – PGRN *in vivo* models

We successfully constructed $Grn^{-/-}$ homozygous knockout mice using a targeted, homologous recombination approach. The mouse *Grn* gene, located on chromosome 11, extends over 6.5 kb. An approximately 11.7 kb fragment including the *Grn* gene from a *Grn* specific 129/Sv BAC clone from a mouse bMQ BAC library (Sanger Institute) was subcloned into a pZero™-1 vector. Using targeted genomic mutagenesis, two *Lox*P sites were inserted flanking *Grn* exons 1 and 3 for eventual deletion of 349 bp of coding sequences encoding for the Start-ATG, signal peptide, and the first granulin domain. As a positive selection marker, we utilized neomycin resistance gene flanked with FRT sites and a *Diphtheria toxin A* (*DTA*) cassette outside the 3' recombination arm for negative selection. The targeting construct was linearized and electroporated into 129/Sv ES cells. Recombinant ES cells were expanded and 2 independent ES cell lines were injected into C57/BL6 blastocysts and transferred into pseudopregnant females. Chimeras were born and bred with constitutive Cre-expressing mice to generate heterozygous $Grn^{-/+}$ mice that were further bred to generate homozygous $Grn^{-/-}$ mice. $Grn^{-/-}$ mice up to 15 months do not show any gross abnormality or increased mortality. Currently these mice

are being analyzed by behavioral, histological and biochemical methods (Wils *et al.*, in preparation).

Consistent with an important role of inflammatory responses in AD, we also identified Grn as one of the top ten up-regulated molecules in mouse models of amyloidosis. On gene expression analysis of laser-microdissected plaque tissue from Tg2576 and APPPS1 mice Grn was upregulated approximately 8-fold in Tg2576 and 2-fold in APPPS1 mice compared to littermate controls. Brain Grn levels in these mouse models correlated significantly with amyloid load, especially the dense-core plaque pathology ($p < 0.001$). We further showed that Grn is up-regulated in microglia and neurons and neurites around dense-core plaques, but not in astrocytes or oligodendrocytes in AD mouse models as well as in AD patients. These data support the ongoing use of these mouse models in anti-inflammatory drug trials with Grn as a disease biomarker on mouse models. Also, a strong GRN reactivity around dense-core plaques is consistent with an important role of these types of plaques in AD pathogenesis (Pereson *et al.*, 2009).

Specific aim 4 – PGRN *in vitro* model systems

As part of experiments proposed under Aim 4, we studied the cell biology of PGRN in context of TAR DNA binding protein-43 (TDP-43), phosphorylated and cleaved forms of which are deposited within intraneuronal inclusions in FTD patients. Utilizing primary cortical neurons derived from Grn-deficient mice, we first showed that Grn deficiency causes significantly reduced neuronal survival accompanied by increased caspase activation and TUNEL reactivity. This phenomenon was unique to cortical neurons because neither siRNA-mediated Grn knockdown in H4 and HeLa cells, nor allelic loss of Grn in primary mouse embryonic fibroblasts (MEFs), showed appreciably increased apoptosis. We further showed that treatment of Grn-deficient neurons and MEFs with the proteasomal blocker MG132 leads to caspase-mediated TDP-43 fragmentation and accumulation of detergent-insoluble 35-kDa and 25-kDa C-terminal fragments (CTFs). We also showed that full-length TDP-43 also accumulated in the detergent-insoluble fraction and although inhibition of caspases prevented MG132-induced generation of TDP-43 CTFs, it did not block the pathological conversion of full-length TDP-43 from soluble to insoluble species. We therefore showed that Grn not only functions as a neuronal survival factor for cortical neurons, but Grn deficiency also causes increased aggregation and accumulation of full-length TDP-43 along and its C-terminal derivatives by both caspase-dependent and independent mechanisms (Kleinberger *et al.*, in submission).

Publications

- Sleegers,K., Brouwers,N., Van Damme,P., Engelborghs,S., Gijselinck,I., van der Zee,J., Peeters,K., Mattheijssens,M., **Cruts,M.**, Vandenberghe,R., De Deyn,P., Robberecht,W., **Van Broeckhoven,C.**: Serum biomarker for progranulin-associated frontotemporal lobar degeneration. Annals of Neurology 65: 603-609 (2009) (PMID: 19288468) (I.F.: 9.935)
- Pereson,S., Wils,H., Kleinberger,G., Van Broeck,B., Joris,G., McGowan,E., Jucker,M., Deforce,D., **Van Broeckhoven,C.**, **Kumar-Singh,S.**: Progranulin expression correlates with dense-core amyloid plaque burden in Alzheimer disease mouse models. Journal of Pathology 219: 173-181 (2009) (PMID: 19557827) (I.F.: 5.121)
- Rollinson,S., Rohrer,J.D., van der Zee,J., Sleegers,K., Mead,S., Engelborghs,S., Collinge,J., De Deyn,P., Mann,D.M.A., **Van Broeckhoven,C.**, Pickering-Brown,Stuart M.: No association of PGRN 3'UTR rs5848 in frontotemporal lobar degeneration. Neurobiology of Aging: Epub (2009) (PMID: 19446372) (I.F.: 5.959)

Theses

PhD Theses

Name Date	Supervisor / Co-supervisor	Title
Daniel Pirici 20-03-2009	Samir Kumar-Singh Christine Van Broeckhoven	Molecular mechanisms of extracellular and intracellular proteinopathy in Alzheimer's disease and frontotemporal dementia

MSc Theses

Name Date	Supervisor / Co-supervisor	Title
Jonathan Janssens	Samir Kumar-Singh Hans Wils	Biochemical and neuropathological characterization of a novel human TDP-43 overexpression mouse model
Quinten Verelst	Marc Cruts Ilse Gijselinck	Identification of genetic defect in a locus for frontotemporal lobe degeneration
Stéphanie Philtjens	Piet Stinissen Marc Cruts Ilse Gijselinck	Pathway-based genetic analyses to detect novel genes associated with frontotemporal lobar degeneration

Professional BSc Theses

Name Date	Supervisor / Co-supervisor	Title
Jan De Ren	Marc Cruts Ilse Gijselinck	Search of novel genetic factors for frontotemporal lobe degeneration by mutation analysis of functional candidate genes

Honors, Prizes & Awards

Honors

- **Christine Van Broeckhoven**: Chevalier dans l'Ordre National de la Légion d'Honneur, France, 06/02/2009
- **Christine Van Broeckhoven**: European Ambassador for Creativity and Innovation for Belgium, 2009
- **Marc Cruts**: Certificate of Honor of the Foundation for Alzheimer Research (SAO/FRMA), Belgium, 2009
- **Samir Kumar-Singh:** Certificate of Honor of the Foundation for Alzheimer Research (SAO/FRMA), Belgium, 2009
- **Kristel Sleegers**: Certificate of Honor of the Foundation for Alzheimer Research (SAO/FRMA), Belgium, 2009
- **Julie van der Zee**: Certificate of Honor of the Foundation for Alzheimer Research (SAO/FRMA), Belgium, 2009

Prizes

- **Marc Cruts & Samir Kumar-Singh**: Koning Boudewijn Stichting, De Marie-Thérèse De Lava Prijs 2009, 26/11/2009
- **Kristel Sleegers & Marc Cruts**: Nationale Alzheimer Liga, De Santkin Prijs 2009, 21/09/2009

Awards

- **Ilse Gijselinck**: Travel Award of the AD/PD 2009 organizers to attend the 9th International Conference on AD/PD 2009, Prague, Czech Republic, March 11-15, 2009
- **Sandra Pereson**: Travel Award of the Alzheimer's Association to attend the 12th Alzheimer's Association International Conference on Alzheimer's Disease (ICAD) 2009, Vienna, Austria, July 11-16, 2009

Activities & Presentations

Chair and Organizational Activities

International

- **Samir Kumar-Singh:** Symposium Chair, International Congress of Vascular Behavioral and Cognitive Disorders (Vas-Cog): Chair of plenary session II: Animal models and VCI, Singapore, January 14-16 (2009)
- **Samir Kumar-Singh:** Representative from University of Antwerp on Board of the Cyttron Consortium, University of Leiden, The Netherlands (2009)
- **Christine Van Broeckhoven:** Co-Chair of the Scientific Program Committee, International Conference on Alzheimer's disease and related disorders (ICAD): Vienna, Austria, July 11-16 (2009)
- **Christine Van Broeckhoven:** Symposium Chair, International Conference on Alzheimer's disease and related disorders (ICAD), Vienna, Austria, July 11-16 (2009)
- **Kristel Sleegers**: Symposium Chair, 6th European Meeting on Molecular Diagnostics, Scheveningen, The Netherlands, October 21-23 (2009)

National

- **Samir Kumar-Singh**: Co-Chair Thematic Workshop: 'Molecular morphology and neuropathology', Annual IAP P6/43 Meeting, Brussels, Belgium, October 26 (2009)
- **Samir Kumar-Singh**: Symposium Chair, VIB Science Club Neurodegenerative Diseases II, Leuven, October 30 (2009)

Invited Lectures

International

- **Samir Kumar-Singh**: Pre-Congress Special Symposium of Vascular Behavioral and Cognitive Disorders Congress: 'Molecular pathology of vascular dementia', Singapore, January 13 (2009)
- **Samir Kumar-Singh**: CAA and familial forms of dementia, 4th International Congress of Vascular Behavioural and Cognitive Disorders (Vas-Cog), Singapore, January 14-16 (2009)
- **Christine Van Broeckhoven:** The role of progranulin (PGRN) in brain function and neurodegenerative disease. 9th International Conference on Alzheimer's & Parkinson's Diseases: AD/PD 2009, Prague, Czech Republic, March 11-15 (2009)
- **Samir Kumar-Singh**: Spectrum of parenchymal and vascular amyloidosis, VIII Congress of the Society of Morphology, Craiova, May 27-30 (2009)
- **Kristel Sleegers**: Serum Progranulin Levels in the Diagnosis of Progranulin-Related Neurodegenerative Diseases. 12th International Conference on Alzheimer's Disease (ICAD) Vienna, Austria, July 11-16 (2009)
- **Christine Van Broeckhoven:** Exploring the brain: from function to dysfunction: molecular genetics of neurodegenerative dementias. The Amsterdam International Medical Summer School 20th Edition, Amsterdam, The Netherlands, July 17 (2009)

National

- **Samir Kumar-Singh:** Plaques and vascular Aβ in Alzheimer's disease, van Leeuwenhoek Lecture, University of Leiden, The Netherlands, January 30 (2009)
- **Christine Van Broeckhoven**: Over dementie: Een gesprek met Professor Christine Van Broeckhoven. Dementie Café Grimbergen, Grimbergen, Belgium, February 18 (2009)
- **Christine Van Broeckhoven**: Genetica van de ziekte van Alzheimer. Vormingplus – Volkshogeschool Gent-Eeklo, Oosterzele, Belgium, February 27 (2009)

- **Samir Kumar-Singh**: CAA associated with familial forms of dementia and mouse models. VIB Seminar 2009, Blankenberge, Belgium, March 12 (2009)
- **Kristel Sleegers**: Serum Progranulin Levels in the Diagnosis of Progranulin-related Neurodegenerative Diseases. Focus Conferences – Spring conference Focus Diagnostica. Anderlecht, Belgium, April 21 (2009)
- **Christine Van Broeckhoven**: The role of progranulin in the genetic etiology of frontotemporal lobe degeneration and related disorders. Seminar Universiteit Luik, Luik, Belgium, April 22 (2009)
- **Christine Van Broeckhoven**: De ontdekking van progranuline in dementie. Studium Generale in de Biomedische Wetenschappen – UA, Antwerpen, Belgium, April 27 (2009)
- **Christine Van Broeckhoven**: Brein en branie – een pionier in Alzheimer. Vormingplus regio Mechelen & cultuurcentrum De Mol in Lier, Lier, Belgium, May 13 (2009)
- **Christine Van Broeckhoven**: Brein en branie. Gezinsbond, Antwerpen, Belgium, May 18 (2009)
- **Samir Kumar-Singh:** Molecular neuropathology of dementias, University of Medicine and Pharmacy Craiova, DOLJ Medical Association, Craiova, May 26 (2009)
- **Christine Van Broeckhoven**: Geheugen en Alzheimerdementie, hoe draagt erfelijkheid bij? Praatcafé 'Dementie' Bree – Bocholt – Meeuwen-Gruitrode, Meeuwen-Gruitrode, Belgium, June 2 (2009)
- **Christine Van Broeckhoven**: Geheugen en dementie, Lions Club Antwerp Airport, Schilde, Belgium, June 15 (2009)
- **Christine Van Broeckhoven**: The failing brain, Opening BRAI²N (Brain Research center Antwerp for Innovative and Interdisciplinary Neuromodulation), UZA, Antwerpen, Belgium, June 19 (2009)
- **Christine Van Broeckhoven**: Neurogenetics of dementia. Medizinisch Genetisches Zentrum Symposium Neurogenetik im Erwachsenenalter, Munich, Germany, June 27 (2009)
- **Christine Van Broeckhoven**: De Mens voorbij? 20ste Gentse Feestendebatten, Gent, Belgium, July 25 (2009)
- **Christine Van Broeckhoven**: Vergrijzing, geheugen en dementie. Rotary Meise-Bouchout, Wolvertem, Belgium, September 2 (2009)
- **Christine Van Broeckhoven**: Vergrijzing, geheugen en dementie. Soroptimist Club Brussel Iris, Brussel, September 16 (2009)
- **Christine Van Broeckhoven**: Jongdementie. Werelddag Dementie, Turnhout, Belgium, September 19 (2009)
- **Christine Van Broeckhoven**: De ziekte van Alzheimer - Recente wetenschappelijke ontdekkingen. VormingPlus & de Vlaamse Alzheimer Liga, Scheldewinkede-Oosterzele, September 25 (2009)
- **Christine Van Broeckhoven**: Brein en Branie. De Uil van Minverva, Bilzen, September 30 (2009)
- **Christine Van Broeckhoven**: Biologie van (Alzheimer-) dementie. Minisymposium: (Alzheimer-)dementie en beslissingen bij het levenseinde, UA, Antwerpen, October 3 (2009)
- **Christine Van Broeckhoven**: Vergrijzing en dementie. Pluralistische Vereniging van Senioren, Hof van Aragon, Lier, October 5 (2009)
- **Kristel Sleegers**: Molecular Genetics of Frontotemporal Dementia. 15th Annual Meeting of the German Society of Neurogenetics, Homburg/Saar, Germany, October 8-10 (2009)
- **Christine Van Broeckhoven**: Genetica van de ziekte van Alzheimer, Zorg-Saam – Ik verlies elke dag (Studiedag over dementie), Wijgmaal, Belgium, October 9 (2009)
- **Christine Van Broeckhoven**: De ziekte van Alzheimer. Buitenbeentjes 2009 - Bibliotheken van het Waasland: Thema – Mens-en-kennis, Sint-Gillis-Waas, Belgium, October 12 (2009)
- **Christine Van Broeckhoven**: Dementie. Gemeentelijke Ouderenadviesraad – Gemeente Steenokkerzeel, Steenokkerzeel, Belgium, October 14 (2009)
- **Marc Cruts:** The Alzheimer Disease & Frontotemporal Dementia Mutation Database. Human Variome Project Meeting 2009 – Spotlight on Neurogenetics, Honolulu Hawai, October 19 (2009)
- **Christine Van Broeckhoven**: Molecular genetics of frontotemporal lobar neurodegeneration. 10ème Réunion Francophone sur la maladie d'Alzheimer et les syndromes apparentés, Nantes, France, October 20-22 (2009)
- **Samir Kumar-Singh**: Wild-type human TDP-43 overexpression in transgenic mice causes motor neuron degeneration. Annual IAP P6/43 Meeting, Brussels, Belgium, October 26 (2009)
- **Christine Van Broeckhoven**: De ziekte van Alzheimer. Causerie Koninklijk Atheneum Sint-Truiden, Sint-Truiden, Belgium October 27 (2009)
- **Christine Van Broeckhoven**: Alzheimer, vergrijzing en emancipatie. Openbare Bibliotheek Huizen-Laren-Blaricum, Huizen, The Netherlands, October 28 (2009)
- **Samir Kumar-Singh**: A Novel ALS Mouse Model with TDP-43 Neuronal Inclusions. VIB Science Club Neurodegenerative Diseases II, Provinciehuis Leuven, Belgium, October 30 (2009)
- **Christine Van Broeckhoven**: Wetenschap en dementie: hoop of hype? Vitamine Q – Staden, Staden, Belgium, November 13 (2009)
- **Christine Van Broeckhoven**: Debat: Vrouw in de wetenschap. 20 jaar Medical Women Association Belgium, Brussel, Belgium, November 14 (2009)

- **Samir Kumar-Singh:** Mouse models of FTLD and ALS, Maastricht Neuroscience Seminar Series, University of Maastricht, The Netherlands, November 17 (2009)
- **Christine Van Broeckhoven**: Genetica van de ziekte van Alzheimer. Bibliotheek Ter Elst te Gistel, Gistel, Belgium, November 18 (2009)
- **Christine Van Broeckhoven**: Stand van zaken van de wetenschap mbt de dementieproblematiek. Vlaamse Alzheimer Liga & Familiegroep in Asse, Asse, Belgium, November 23 (2009)
- **Christine Van Broeckhoven**: Jong-Dement. Euregionale bijeenkomst 'Jong-Dement' van politici/beleidsmakers, Provinciehuis Maastricht, The Netherlands, November 25 (2009)
- **Christine Van Broeckhoven**: Geheugen en dementie. Bibliotheek De Eendracht te Dessel, Dessel, Belgium, November 30 (2009)
- **Christine Van Broeckhoven**: Dementie en Alzheimer. Het Paleis (in samenwerking met Vlaams-Nederlands huis de Buren), Antwerpen, Belgium, December 15 (2009)

Oral Presentations and Slide Sessions

International

- **Ilse Gijselinck:** Linkage to chromosome 9p21 in a Belgian frontotemporal lobar degeneration family with motor neuron disease. Neurodegenerative Diseases 6(S1): 1100 (2009), 9th International Conference AD/PD - Alzheimer's and Parkinson's Diseases: Advances, Concepts and New Challenges, Prague, Czech Republic, March 11–15 (2009)
- **Samir Kumar-Singh**: Fractal Analysis of amyloid plaques in Alzheimer's disease patients and mouse models. Alzheimer & Dementia 5 (4-Suppl 1): 107 (O2-03-01 (2009), 12th International Conference on Alzheimer's Disease (ICAD) Vienna, Austria, July 11-16 (2009)
- **Samir Kumar-Singh:** Overexpression of wild-type TDP-43 Leads To Motor Neuron Degeneration and ALS-like phenotype in germline transgenic mice (Hot Session Symposium II): 12th International Conference on Alzheimer's Disease (ICAD) Vienna, Austria, July 11-16 (2009)
- **Sandra Pereson**: Progranulin correlates with dense-core plaque burden in Alzheimer's disease mouse models. Alzheimer & Dementia 5 (4-Suppl 1): 83 (O1-03-07) (2009), 12th International Conference on Alzheimer's Disease (ICAD) Vienna, Austria, July 11-16 (2009)
- **Kristel Sleegers**: Serum progranulin is a noninvasive biomarker for frontotemporal lobar degeneration. Neurodegenerative Diseases 6(S1): 1021 (2009), 9th International Conference AD/PD - Alzheimer's and Parkinson's Diseases: Advances, Concepts and New Challenges, Prague, Czech Republic, March 11–15 (2009)
- **Julie van der Zee**: A multigenerational family with inherited pathologically confirmed Creutzfeldt-Jakob disease unexplained by PRNP. Alzheimer & Dementia 5 (4-Suppl 1): 164 (O4-06-08) (2009), 12th International Conference on Alzheimer's Disease (ICAD) Vienna, Austria, July 11-16 (2009)
- **Hans Wils**: Biochemical characterization of FTLD-TDP overexpression and knockout mouse models. Alzheimer & Dementia 5 (4-Suppl 1): 154 (O4-03-03) (2009), 12th International Conference on Alzheimer's Disease (ICAD) Vienna, Austria, July 11-16 (2009)

National

- **Tim Van Langenhove**: Clinical heterogeneity in two unrelated families linked to the valosin-containing protein p.R159H mutation. 8th Bi-annual Meeting of the Belgian Society for Neuroscience, Liège, Belgium, May 11 (2009)
- **Samir Kumar-Singh:** Molecular neuropathology of FTLD, Neurology Research Club, Academic Hospital of Antwerpen, June 12, 2009
- **Sandra Pereson:** Role of progranulin in an Alzheimer disease. Annual Scientific IAP P6/43 meeting, Brussels, Belgium, October 26 (2009)
- **Tim Van Langenhove**: Genetic contribution of FUS to Frontotemporal Lobar Degeneration. Annual Scientific IAP P6/43 meeting, Brussels, Belgium, October 26 (2009)
- **Samir Kumar-Singh:** Molecular Pathology of Alzheimer's disease and frontrotemporal lobar degeneration Onderzoeksdag – Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen, October 30 (2009)

Poster Presentations

International

- **Nathalie Brouwers**: No major role for TARDBP in Alzheimer genetic etiology. Neurodegenerative Diseases 6(S1): 950 (2009), 9th International Conference AD/PD - Alzheimer's and Parkinson's Diseases: Advances, Concepts and New Challenges, Prague, Czech Republic, March 11–15 (2009)
- **Kleinberger,G.**: Survival apoptosis and characterization of TDP-43 in cells derived from progranulin knockout mice. Alzheimer & Dementia 5 (4-Suppl 1): 444-445 (P4-046) (2009), 12th International Conference on Alzheimer's Disease (ICAD) Vienna, Austria, July 11-16 (2009)

National

- **Nathalie Brouwers**: No major role for TARDBP in Alzheimer genetic etiology. Annual Scientific IAP P6/43 meeting, University of Brussels (ULB), Brussels, Belgium, October 26 (2009)
- **Ilse Gijselinck**: A genome-wide linkage study in a multiplex FTLD-ALS family identifies two loci at chromosomes 9 and 14. Annual Scientific IAP P6/43 Meeting, University of Brussels (ULB), Brussels, Belgium, October 26 (2009)
- **Gernot Kleinberger**: Increased apoptosis in cortical cultures derived from progranulin knockout mice. Annual Scientific IAP P6/43 Meeting, University of Brussels (ULB), Brussels, Belgium, October 26 (2009)
- **Gernot Kleinberger**: Increased apoptosis in cortical cultures derived from progranulin knockout mice. VIB Science Club Neurodegenerative Diseases II, Leuven, Belgium, October 30 (2009)
- **Sandra Pereson**: The role of progranulin in Alzheimer Disease. VIB Science Club Neurodegenerative Diseases II, Leuven, Belgium, October 30 (2009)
- **Hans Wils**: Wild-type human TDP-43 overexpression in transgenic mice causes an ALS-like motor neuron degeneration with associated neuronal inclusions. Annual Scientific IAP P6/43 Meeting, University of Brussels (ULB), Brussels, Belgium, October 26 (2009)
- **Tim Van Langenhove**: Genetic contribution of FUS to Frontotemporal Lobar Degeneration. VIB Science Club Neurodegenerative Diseases II, Leuven, Belgium, October 30 (2009)
- **Hans Wils**: Biochemical characterization of FTLD-TDP overexpression and knockout mouse models. VIB Science Club Neurodegenerative Diseases II, Leuven, Belgium, October 30 (2009)

Progress report of the research group of

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Functional analysis of novel adhesive and signaling proteins in development and tumorigenesis of neural tissues

1. Overview and mission statement

The **research topics** of the Molecular Cell Biology group at the Department for Molecular Biomedical Research (Ghent University - VIB) in the field of neurosciences are the following:
- functional analysis and manipulation of selected catenin genes in neural tissues;
- structure-function analysis of selected protocadherin genes;
- structure-function analysis of the completely new NBPF gene family with putative tumor suppressor activity in neural tissues.

Briefly, **catenins** are proteins that bind to the cytoplasmic domains of classic cadherins, which are well-known cell-cell adhesion molecules. They often form a physical bridge between cadherins and the cytoskeleton, but they can also be involved in cytoplasmic and intranuclear signaling processes. Catenin molecules we focus on are **αT-catenin**, which is related to the epithelial **αE-catenin** but is expressed also in the brain, and catenin p120 (**p120ctn**), which is a so-called Armadillo protein that is expressed in numerous isoforms, some of which are predominant in the brain.

Protocadherins are transmembrane proteins that differ in various aspects from classic cadherins. They are expressed predominantly in the brain, but their functions are largely unexplored. We are particularly interested in the delta-protocadherins.

NBPF (Neuroblastoma BreakPoint gene Family) is a new gene family that is presumably involved in **suppression** of **neuroblastoma**, a malignant tumor from undifferentiated neuroectodermal cells derived from the neural crest. We discovered the first member of this gene family at a balanced chromosomal breakpoint in a neuroblastoma patient. This gene family, which now includes 22 members, is intricate structurally and possibly functionally as well. Our current documented hypothesis is that NBPF proteins are candidate tumor suppressors involved in signaling processess in the cytoplasm.

2. Research on Catenins

2.A. αT-catenin: a novel α-catenin with tissue-restricted expression

Classic cadherins interact homophilically via their extracellular domains, functioning as physical linkers between adjacent cell membranes. The cytoplasmic region of classic cadherins binds β-catenin, which in turn associates with **α-catenin**. Alpha-catenin is indispensable for cadherin-mediated cell adhesion. In the absence of α-catenin, the intercellular junctions are strongly affected, and in the case of tumors derived from epithelial cells this has severe consequences in the form of increased malignancy (Vermeulen et al., 1999).

Additional complexity exists, as there are three homologous α-catenin proteins: the rather ubiquitously expressed αE-catenin (Herrenknecht et al., 1991; Nagafuchi et al., 1991), neural αN-catenin (Hirano et al., 1992), and **αT-catenin** (Janssens et al., 2001). Loss of αN-catenin affects the stability of dendritic spines and synaptic contacts between neurons (Abe et al., 2004). For αT-catenin, we have demonstrated

that it has a restricted expression pattern: from very strong expression in heart tissue, where it is co-expressed with αE-catenin at intercalated discs, to strong expression in the peritubular myoid cells of testis, and modest expression in skeletal muscle and brain. Using antibodies raised 'in house' we showed localization of αT-catenin at specific cortical layers of the brain and in the molecular layer of the cerebellum. The mRNA of αT-catenin was detected in the granular layer (Vanpoucke et al., 2004) However, the function of αT-catenin in the brain remains poorly understood.

Human αT-catenin is encoded by *CTNNA3* (also called *VR22*). As this gene is positioned within a chromosome-10 region that has been linked to particular cases of familial **Alzheimer's disease** (AD), we assessed in collaboration the possible involvement of *CTNNA3* in this disabling disease. We showed that αT-catenin can inhibit Wnt signaling and meets the criteria for both a positional and a functional candidate for AD susceptibility (Busby et al., 2004). However, none of the *CTNNA3* SNPs in our study appeared to be strongly associated with chromosome-10-linked AD (Busby et al., 2004), whereas another study suggested the contrary (Ertekin-Taner et al., 2003). Whether particular variants or mutations of αT-catenin influence susceptibility to AD remains a matter of debate (Bertram et al., 2007; Martin et al., 2005; Miyashita et al., 2007)

Recently we were able to produce a mouse in which αT-catenin is ablated by conditional gene knock out (KO) (see Fig. 1A and B). We are now studying this mouse anatomically, histologically, physiologically, and behaviorally. This will allow us to prove or disprove whether αT-catenin plays an important role in neural tissues. For instance, the granular layer of the cerebellum of these KO mice shows fewer and poorly organized cell bodies (exemplified in Fig. 1C and D). We also observed a behavioral phenotype in these KO mice. We need to determine whether this is caused by a muscular or a neural defect.

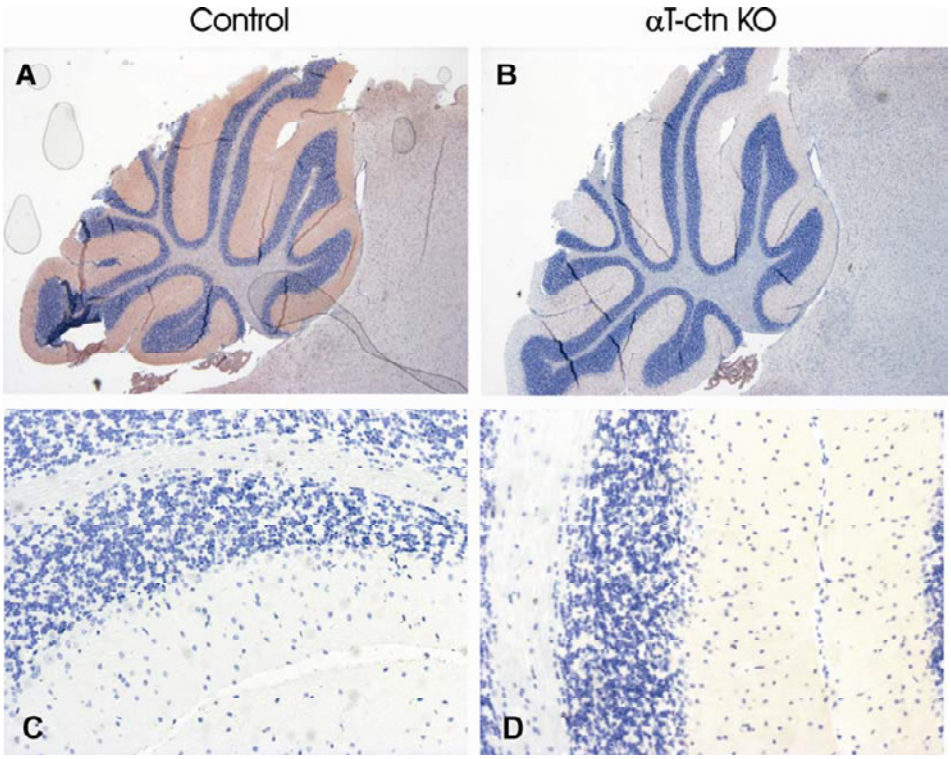

Figure 1: Cerebellum of αT-catenin KO mouse compared to WT mouse (8 months old). (A, B) Immunohistochemical staining for αT-catenin in molecular layer of the cerebellum is greatly reduced in the αT-catenin KO mouse compared to wild type mouse. (C, D) Cell density and organization of cell bodies is affected in the granular layer of the cerebellum of KO mouse compared to wild type mouse.

2.B. Functional analysis of p120ctn isoforms in the brain

The aim of this part of the project was to study the role of **p120catenin** (p120ctn) isoforms in the brain using a transgenic approach. **Alternative splicing** of the human p120ctn gene *(CTNND1)* gives rise to 48 possible p120ctn isoforms originating from four start codons and four alternatively used exons (Keirsebilck et al., 1998). The alternatively used exon C encodes six amino acids that interrupt a nuclear localization sequence *(NLS)*. This interruption abrogates the p120ctn inhibition of RhoA, suggesting that the p120ctn isoform C acts as a physiological regulator of RhoGTPase activity in the brain. Indeed, RT-PCR and Q-PCR have shown strong expression of p120ctn isoform C in the brain. To assess the *in vivo* function of exon C, we generated p120ctn **exon-C-specific knock-out *(p120 KO-C)* and knock-in *(p120 KI-C)* mice**. Surprisingly, both KO-C/KO-C and KI-C/KI-C mice died as early as the blastocyst stage (3.5 dpc).

We are now analyzing the functionality of p120ctn isoform C beyond the developmental stage. For this purpose we used **p120 fl/fl** mice, in which exons with all four possible start codons are flanked by loxP sites (provided by Dr. A. Reynolds, Nashville, USA) (Davis and Reynolds, 2006). These mice were crossed with **Nestin-Cre mice** (Tronche et al., 1999), resulting in offspring lacking all p120ctn isoforms in the cerebellum and in all cortical layers of the brain. These KO mice showed higher levels of active RhoA in the brain compared to control mice whereas active Rac1 levels were unchanged. Nonetheless, the brain-confined p120ctn knock-out mice turned out to be viable and to develop brains of normal size and overall normal anatomy and histology.

To examine the influence of p120ctn exon C on the brain phenotype, we crossed the p120ctn fl/fl x Nestin-Cre mice with p120 KO-C/wt and p120 KI-C/wt mice. The offspring had a constitutive ablation/insertion of p120ctn exon C in one allele in combination with a brain-specific deletion of all p120ctn isoforms in the other allele. Both KOC/fl;Nestin-Cre and KIC/fl;Nestin-Cre mice were viable. Histological analysis of KOC/fl;Nestin-Cre mice revealed a medial hippocampal abnormality (Fig. 2A) that was not obvious more laterally (Fig. 2B).

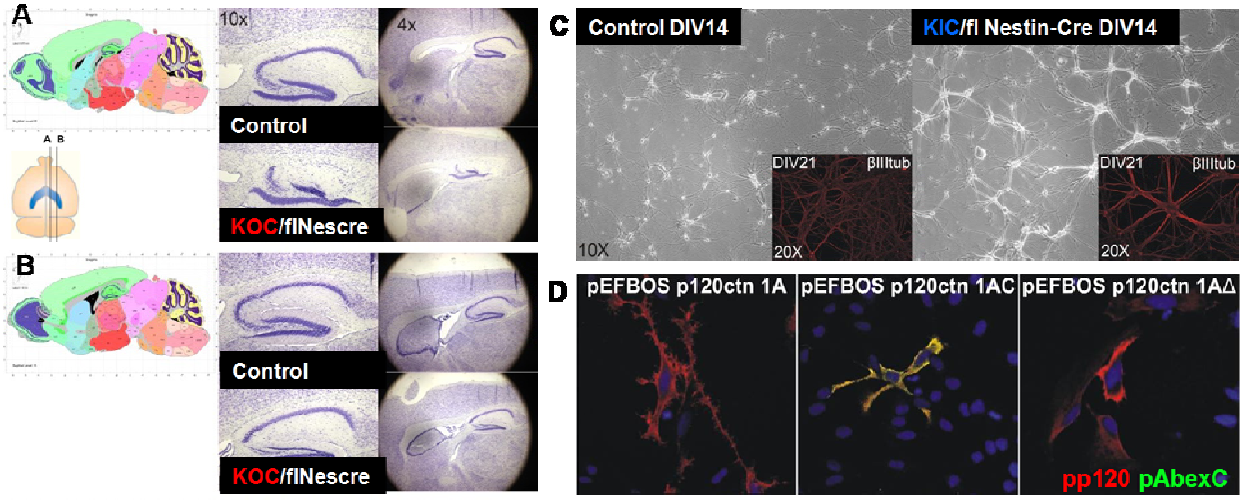

Figure 2. Analysis of p120ctn KOC/fl;Nestin-Cre and KIC/fl;Nestin-Cre mice. KOC/fl;Nestin-Cre mice show a medial (A), but not a lateral (B) hippocampal defect. KIC/fl;Nestin-Cre hippocampal cultures show a fasciculation phenotype (C) compared to control cultures. Inset: staining for neuronal marker βIII tubulin. (D) NIH3T3 cells transfected with p120ctn isoform 1A, p120ctn isoform 1AC or RhoA-uncoupled p120ctn (p120ctn 1AΔ), and stained for all p120ctn isoforms (pp120) and for p120ctn isoform C (pAbexC).

Microcephaly was observed in KIC/fl;Nestin-Cre mice, but this phenotype was not fully penetrant. Neuronal cultures derived from KIC/fl;Nestin-Cre hippocampi showed a predominant fasciculation phenotype compared to control hippocampal cultures (Fig. 2C). These data are in line with our *in vitro* results. Overexpression of p120ctn isoform 1A, which is the most abundant p120ctn isoform in the brain, led to a dendritic branching phenotype (Fig. 2D *left*) (Reynolds et al., 1996). In contrast, p120ctn isoform 1AC expression inhibited this branching phenotype (Fig. 2D *middle*) to a similar extent as a RhoA-uncoupled p120ctn mutant (Fig. 2D *right*). This reflects the ability of p120ctn isoforms to modulate the activity of **RhoGTPases**, which are known to influence normal dendritic spine density and morphology (Anastasiadis et al., 2000). Abnormal spine morphology is also seen in patients with nonsyndromic mental retardation and cognitive disorders (Govek et al., 2005). We are now investigating the RhoA status in the brains of our different mouse models and trying to dissect how RhoGTPase signaling in hippocampus-derived neuronal cultures is affected by using constitutively active or dominant-negative RhoA mutants, or by using pharmacological inhibitors and activators. Suitable assays will be used to assess potential behavioral alterations in these unique mouse lines. By determining the function of p120ctn and more particularly its isoform C in the brain, we hope to contribute to the understanding of particular human neurological disorders at the molecular level.

2.C. Functional analysis of p120ctn in neural crest cell development

Several cadherins play important roles and display dynamic spatial and temporal expression patterns during development of neural crest cells (NCC) (Taneyhill, 2008). NCC are pluripotent migratory cells arising from the dorsal neural tube via an epithelial-to-mesenchymal transition (Hay, 1995; Thiery, 2003). Disruption of normal cadherin function results in aberrant NCC delamination from the neural fold, migration abnormalities, or defective reaggregation after homing (Borchers et al., 2001; Bronner-Fraser et al., 1992; Luo et al., 2006). It is known that p120ctn plays an important role in regulating the adhesive strength of cadherins by inhibiting their degradation and controlling their level in cell junctions (Davis et al., 2003; Xiao et al., 2003).

The aim of this part of the project was to explore the function of p120ctn during NCC development. For that purpose we crossed p120 $^{fl/fl}$ mice (Davis and Reynolds, 2006) with Wnt1Cre mice (Danielian et al., 1998) to generate knockout mice in which p120ctn is specifically deleted from NCC. We showed that deficiency of p120ctn in NCC results in complex malformations of the ocular anterior segment structures, including corneal opacification, loss of iridocorneal angle and anterior chamber, corneal malformation, and hypoplastic iris and ciliary body. In addition, the mutant mice finally develop glaucoma because of loss of trabecular meshwork and Schlemm's canal. Previous *in vivo* fate mapping experiments revealed that NCC contribute substantially to the eye (Ittner et al., 2005). Expression analysis of p120ctn by immunohistochemistry at the E13.5 and P1 stages in p120$^{fl/fl}$;Wnt1Cre mice showed that its expression in developing ocular mesenchyme cells, corneal stroma and endothelium, and iridoconeal angle was substantially reduced in comparison to wild type mice (Fig. 3A).

To determine if the ocular defects are due to impaired NCC migration or to a differentiation defect, we introduced the Rosa26 reporter (Rosa26R) allele (Soriano, 1999) into mice with p120ctn floxed alleles. Then, by crossing with Wnt1Cre lines, the fate of eye cells with p120ctn ablation could be monitored in the progeny by histochemical staining for β-galactosidase. We took p120ctn$^{fl/+}$;Wnt1Cre;Rosa26R mice as control because they displayed no eye defects. We stained eyes at different stages but did not find any evidence for severe defects in either NCC genesis or NCC migration in p120$^{fl/fl}$;Wnt1Cre mice (Fig. 3B). Therefore, ocular defects in p120$^{fl/fl}$;Wnt1Cre mice might be due to a differentiation failure after correct migration and homing.

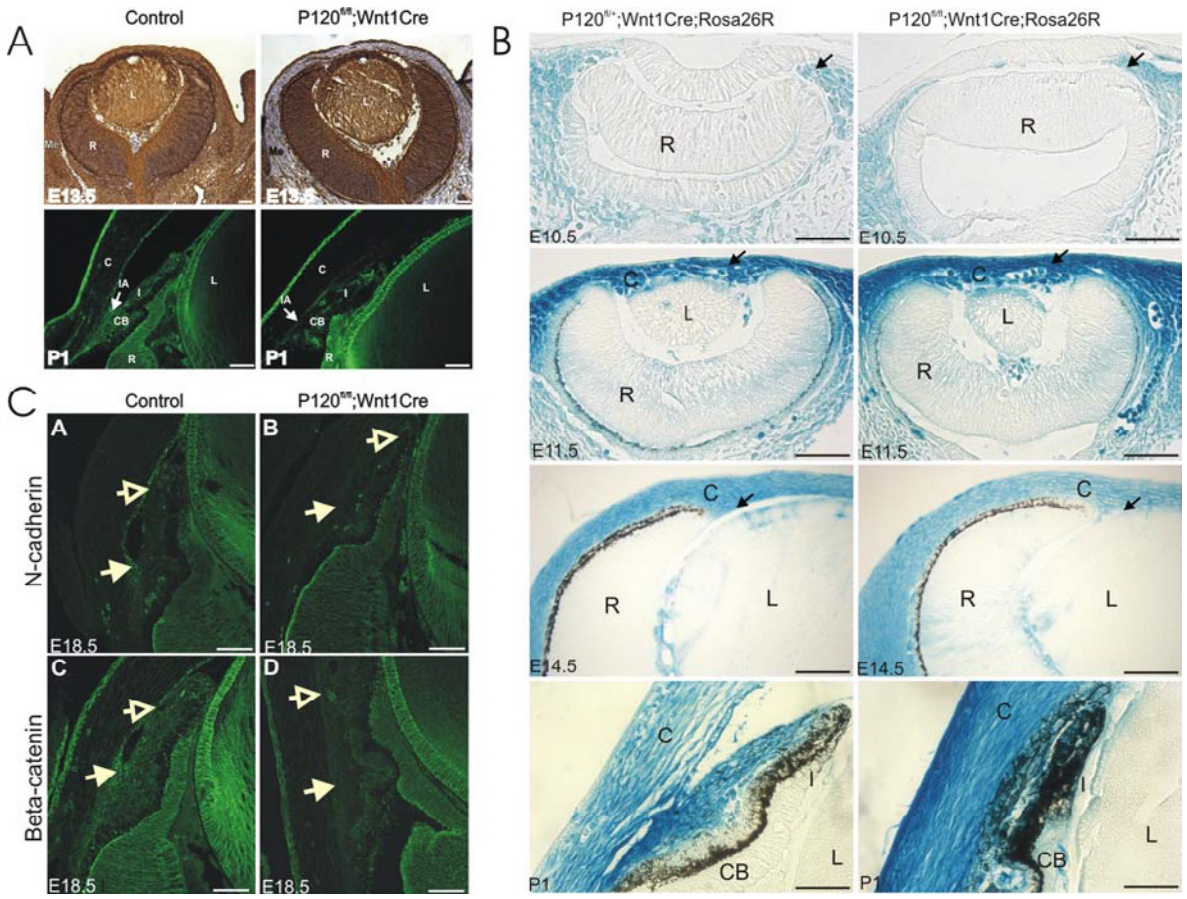

Figure 3. Analysis of eyes in mice with specific ablation of p120ctn in neural crest (NC) progenitor cells. (A) Analysis by immunostaining of the expression pattern of p120ctn in the developing eye. This indicated successful depletion of p120ctn in neural crest (NC)-derived ocular cells in p120$^{fl/fl}$;Wnt1Cre mice. (B) *In vivo* fate mapping of NC-derived ocular cells shows no defect in genesis or migration of NCC in p120$^{fl/fl}$;Wnt1Cre mice. (C) N-cadherin and β-catenin are downregulated in eyes of p120$^{fl/fl}$;Wnt1Cre mice compared to heterozygous p120$^{fl/+}$;Wnt1Cre control mice.

To investigate the mechanism underlying the ocular defect in p120$^{fl/fl}$;Wnt1Cre mice, we analyzed the expression levels of different cadherins and their associated catenins. Immunofluorescence staining revealed that the level of N-cadherin in mutant eyes was lower in the corneal iridocorneal and angle endothelium than in control eyes (Fig. 3C). Similarly, expression of β-catenin was also downregulated in the iridocorneal angle and corneal endothelium in mutant mice (Fig. 3C). Therefore, abnormal cell sorting following N-cadherin dysregulation could be the basis of the ocular defects. We are now further exploring the mechanism underlying the eye defects at the cell level.

3. Role of Delta-Protocadherins in Neural Tissues

With more than 80 different protocadherin (Pcdh) genes in man and mouse and an increasing number of splice variants, these genes comprise a major group within the cadherin gene superfamily (Hulpiau and van Roy, 2009). They differ from classic cadherin genes in numerous aspects and are expressed predominantly in neural tissues (Redies et al., 2005). Protocadherins can be divided into clustered α-, β- and γ-protocadherin genes, and nonclustered δ-protocadherin (δ-Pcdhs) genes. In the present project we aim to characterize selected human δ-protocadherins (δ-PCDHs) and their mouse orthologs and to examine their roles in brain development and neurological disorders such as mental illness, dementia

and brain tumors. Part of our research strategy is to conditionally knock out the *Pcdh10* gene in the mouse, and another part is to ectopically express different isoforms of human PCDH11, also in the mouse.

It was recently demonstrated that mice with total *Pcdh10* knockout in the germline develop severe defects in growth of striatal axons and thalamocortical projections (Uemura et al., 2007). These mice die within several weeks after birth, which prevents functional and pathological studies at an older age. To avoid this early death, we started the generation of conditional *Pcdh10* knockout mouse models that allow deletion of Pcdh10 in a tissue- and time-specific manner. On the one hand, we are establishing a model in which all isoforms of Pcdh10 can be knocked out by the Cre-LoxP technology (Fig. 4A).

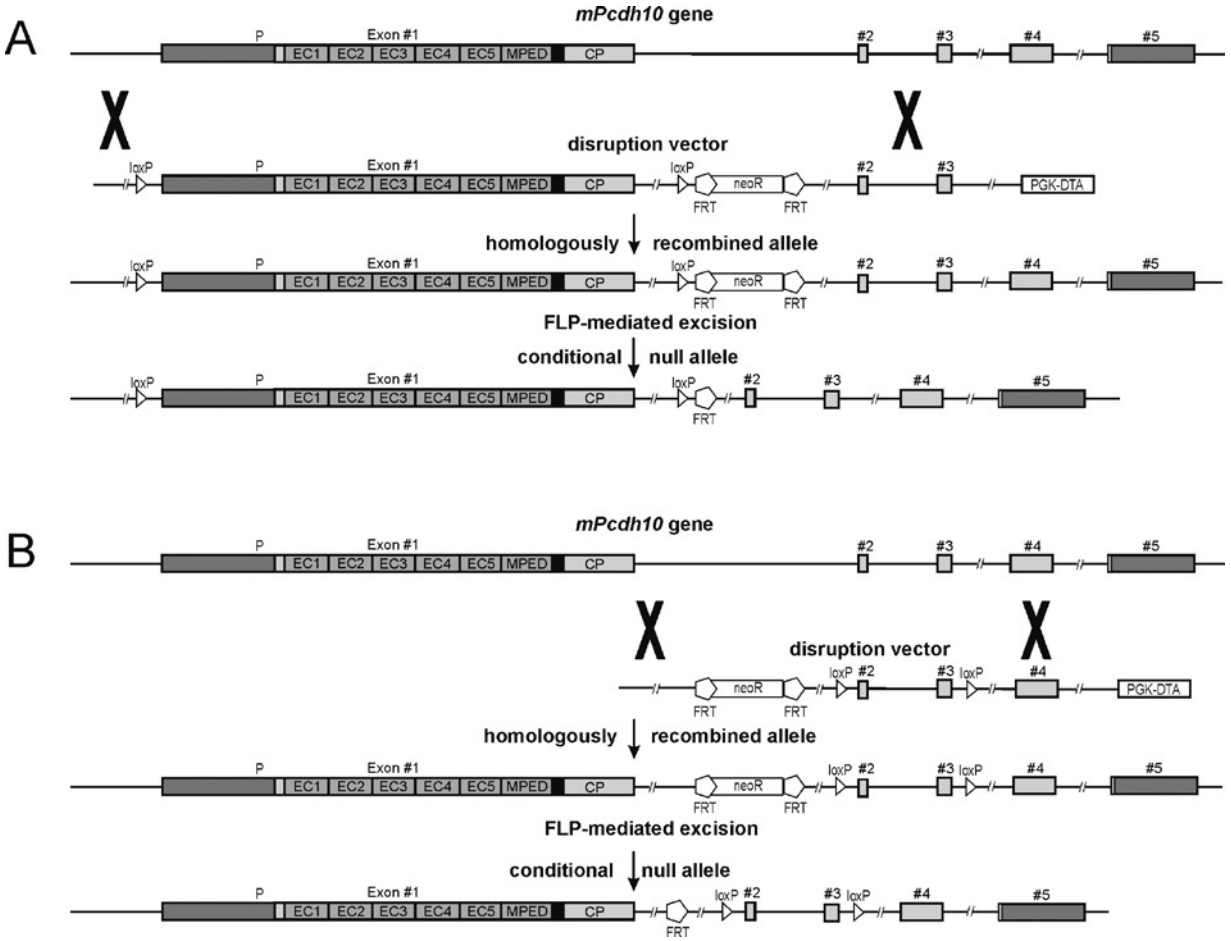

Figure 4. Construction of *Pcdh10* knockout alleles affecting either all isoforms of protocadherin-10 (A) or only the long isoform (B). Embryonic stem (ES) cells with a homologously recombined *Pcdh10* allele can be selected by using a positive selection marker (neoR gene) in combination with loss of the negative selection marker (DTA, diphtheria toxin subunit A). A floxed, conditional null *Pcdh10* allele is obtained upon excision of the neo^R gene by the action of the FLP recombinase at FRT sites. Action of Cre recombinase on the floxed alleles deletes most of the PCDH10 protein in (A) but only the elongated cytoplasmic tail of the longer isoform in (B). The latter tail comprises the CM1 and CM2 conserved motifs.

On the other hand, we are generating a mouse model in which only the long isoforms of Pcdh10 are conditionally knocked out, resulting in lack of the conserved cytoplasmic domains CM1 and CM2 in the encoded Pcdh10 proteins (Fig. 4B). This model will be used to explore the role of these conserved domains in various intracellular signaling pathways. These mice with various floxed parts of the *Pcdh10* gene will then be crossed with different Cre mice in order to elucidate the role of Pcdh10 in important processes, such as controlled cell proliferation, cell migration, cell differentiation, and programmed cell death.

Besides studying δ-protocadherins during mouse development, we are trying to interpret their functions during development of the frog *Xenopus tropicalis* development. For this purpose, we first cloned the cDNA probes for each gene and analyzed the expression patterns by *in situ* hybridization on different stages of *X. tropicalis* embryos. The results revealed that several δ-protocadherin genes were expressed in the neural tissues of *X. tropicalis*. Both Pcdh10 and Pcdh19 are expressed in the eyes and brain. Pcdh20 is expressed in the olfactory vesicle and otic vesicle. We will investigate the functional implications of the above protocadherins by applying the morpholino (MO)-knockdown technique as well as overexpression experiments. Preliminary data revealed eye defects in the Pcdh10 MO-knockdown embryos, including smaller eyes and disorganized retina and lens. We are currently working on the mechanism underlying this phenotype.

In humans, the closely related δ-protocadherins **PCDH11X** and **PCDH11Y** are encoded by chromosomes X and Y, respectively. PCDH11X has mammalian and vertebrate orthologs but PCDH11Y exists only in man (Wilson et al., 2006). Genetic variation in PCDH11X was most recently reported to be associated with susceptibility to late-onset Alzheimer's disease (Carrasquillo et al., 2009). A cytoplasmic variant of PCDH11Y has been associated with the Wnt signaling pathway and tumor formation (Terry et al., 2006; Yang et al., 2005). To generate informative animal models, we attempt to ectopically and conditionally express human PCDH11X and PCDH11Y in the mouse. For this purpose, we constructed tetracycline inducible constructs complementing the *hprt* locus (to achieve single vector integration). cDNA inducibility by doxycyclin was validated before transfection into hprt-deficient ES cells. Induced proteins were localized via an attached E-tag. In the meantime, the constructs have been transfected and correct vector integrations were confirmed by Southern blot analysis. Chimeric mice were born and found to transmit the transgene to their germline. At present, these mice are being bred to homozygosity in order to cross them with appropriate Cre mouse lines and mouse tumor models.

Because our knowledge of the signaling functions of δ-Pcdhs is quite limited, we recently initiated screenings by Y2H (Yeast 2-Hybrid) and MAPPIT (MAmmalian Protein-Protein Interaction Trap) (Eyckerman et al., 2001) to identify novel intracellular molecules specifically interacting with these Pcdhs. For PCDH11X, three strong and two moderate interactors were identified and the molecular mechanism of interaction and its functional implications are under investigation. One of the strong interactors is dynein light chain 1 (DYNLT1), which is a part of the dynein motor complex but has also been reported to have dynein motor-independent functions (reviewed in Vallee et al., 2004). DYNLT1 has been shown to influence actin dynamics during neurite outgrowth (Chuang et al., 2005) and to have a negative effect on neurogenesis (Gauthier-Fisher et al., 2009). Using mutated or shortened constructs of the PCDH11X cytoplasmic tail and of DYNLT1 in an analytical MAPPIT assay, the interaction domains of the two partners were narrowed down. The results obtained with these constructs were unequivocal and the interaction seemed to be isoform-specific, as it could not be confirmed for PCDH11Y. Furthermore we found that DYNLT1 also interacts with PCDH10. For both PCDH10 and PCDH11X this occurred in a DYNLT1 phosphorylation dependent way. We are currently attempting to identify the functional implication of the PCDH-DYNLT1 complexes by endogenous pulldown experiments, analysis of selected tissue samples, and siRNA-mediated knockdown. We use these approaches routinely in our department. Functional consequences will be assessed by assays of cell aggregation, analysis of neurite extension, evaluation of dendritic spine mobility, etc.

Apart from our functional analyses, we are also interested in the mechanisms controlling the expression of the δ-PCDHs. In one approach, we searched for microRNAs regulating the mRNA stability of the δ-PCDHs. Interestingly, some of the microRNAs predicted to target δ-PCDHs have been shown to

play a crucial role in neural development (Cheng et al., 2009). So far, our experiments identified two microRNAs negatively controlling either *PCDH1* or *PCDH7* expression levels. Future research will focus on detailed characterization of this downregulation, in addition to a complementary approach aiming at identification of microRNAs regulating δ-PCDHs translation instead of mRNA stability.

4. NBPF1: a novel neuroblastoma suppressor gene?

We identified the ***NBPF1* gene** (Neuroblastoma BreakPoint Family member 1) while cloning the **breakpoint** of a constitutional **translocation** t(1;17)(p36.2;q11.2) discovered in a **neuroblastoma** (NB) patient (Laureys et al., 1995; Vandepoele et al., 2008). NBs originate from primitive, pluripotent, sympathetic nerve cells derived from the neural crest. These cells can differentiate into the different normal tissues of the sympathetic nervous system, such as the spinal sympathetic ganglia, Schwann cells, and adrenal chromaffin cells. As a result, NBs develop where these neural cells are normally located, most frequently in the adrenal medulla or in the chest cavity. NBs frequently have aberrations of the chromosomal regions 1p36 and 17q11, which are involved in the translocation that we cloned. Hence, we hypothesized that the t(1;17)(p36.2;q11.2) translocation, which we characterized, predisposed the patient to tumor development due to disruption of the *NBPF1* gene.

By quantitative RT-PCR we showed a decreased level of the *NBPF* transcripts in certain types of neuroblastoma cell lines (Vandepoele et al., 2008). Additionally, expression profiling of the *NBPF1* gene showed that its expression is significantly weaker in cell lines with heterozygous loss of *NBPF1* than in cell lines with a normal 1p chromosome (Vandepoele et al., 2008). A similar tumor-associated downregulation was observed for only 15-20% of the genes located in this region, which suggests that the downregulation of at least some of these genes is functionally involved in neuroblastoma pathogenesis (Janoueix-Lerosey et al., 2004). This points to involvement of additional mechanisms besides loss of heterozygosity in the downregulation of some of these genes.

To investigate the factors controlling the expression of *NBPF1*, we isolated its promoter region. Interestingly, this promoter had been copied from an unrelated gene, *EVI5*, after the divergence between simians and prosimians, but before simian radiation (Vandepoele et al., 2009). Like *NBPF1*, *EVI5* was also first identified by virtue of its disruption by a constitutional translocation in a neuroblastoma patient (Roberts et al., 1998), suggesting that the *NBPF1/EVI5* genes are causally related to neuroblastoma. This link was further strengthened by a recent publication describing how copy number variations in an *NBPF* family member were associated with an increased chance of developing neuroblastoma (Diskin et al., 2009). Furthermore, we have shown that expression of NBPF1 in a human colorectal cell line severely suppressed colony formation in soft agar, demonstrating that NBPF1 might act as a tumor suppressor gene (Vandepoele et al., 2008), at least in colon cancer. So far, technical difficulties have prevented us from testing this hypothesis in neuroblastoma cell lines. As colorectal cancer is also characterized by frequent deletions or translocations of 1p36 (Schwab et al., 1996), we believe that our present data represent a first step in the elucidation of the potential tumor suppressive properties of *NBPF1*.

By searching for **NBPF1-interacting proteins**, we obtained additional evidence that NBPF1 has a role in formation of tumors in the nervous system. For instance, we found that the amino-terminal domain of NBPF1 interacts with the Chibby protein, an antagonist of the Wnt/Wingless pathway, which plays a key role in neural crest development and oncogenesis (Takemaru et al., 2003). However, further functional

investigation of this interaction showed no influence of the binding between Chibby and NBPF1 on Wnt signaling, as measured by TOPFLASH activity. Moreover, no competition between β-catenin and NBPF1 was observed in their interactions with Chibby. This suggests the existence of additional, possibly NBPF-modulated functions for Chibby besides its repressor function in the Wnt pathway (Vandepoele et al., 2009*). These putative functions of Chibby, which are independent of nuclear β-catenin but might involve cytoplasmic NBPF1, await further study. Furthermore, we are also trying to unravel the crystal structure of the NBPF-Chibby complex, which could give us more insight into the structural details of this intriguing molecular interaction.

Apart from investigating the interaction between NBPF1 and Chibby, we are also searching for other NBPF1-binding partners. Using affinity purification of endogenous NBPF1 and mass spectrometric analysis of the proteins in the complex, we identified a particular mitochondrial protein as another putative interaction partner for NBPF1. Although NBPF1 is a cytoplasmic protein and an interaction with a mitochondrial protein therefore appears to be an artifact, we demonstrated that NBPF1 interacts only with the precursor form, which is present in the cytoplasm, and not with the mature processed form, which is present within mithochondria. Therefore, we hypothesize that NBPF1 ensures proper folding of nascent polypeptide chains during protein translation or is required for proper shuttling from the cytoplasm to the mitochondria. We are presently testing different siRNAs against NBPF1 to explore these putative functions in more detail.

As the NBPF1 gene might play an important role in both normal and cancerous neuronal cells, we are currently investigating the role of NBPF1 during the cell cycle. Transient overexpression of NBPF1 cDNA in HEK293T cells followed by flow cytometric analysis revealed that NBPF1-overexpressing cells were blocked in the G1-stage of the cell cycle, and this could be explained by increased expression of the CDK-inhibitor p21. Further experiments are ongoing to further unravel the mechanism of NBPF1-induced cell cycle arrest.

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Progress report of the research group of

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Molecular mechanisms controlling the development and evolution of the cerebral cortex.

The cerebral cortex is one of the most complex and important structures in our brain. 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 completed in 2009 thanks to the Funding of the FMRE/GSKE, providing a link with recently published work and in preparation, as well as its perspectives in the future.

1. An intrinsic mechanism of corticogenesis from embryonic stem cells.

The cerebral cortex consists of several hundreds of different types of neurons, organized into specific cortical layers and areas, that display specific profiles of gene expression, morphology, excitability and connectivity. The molecular mechanisms underlying the generation of such a cellular diversity remain largely unknown, in particular due to the lack of appropriate reductionist models of cortical development.

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., 2008). 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 lineag. ES cell-derived cortical-like progenitors subsequently differentiate into a stereotyped population of neurons, most of which display landmarks of cortical pyramidal neurons, including a glutamatergic phenotype and a pyramidal morphology. Most strikingly, ES cell-derived neurons correspond to distinct subtypes of cortical neurons that expressed layer-specific markers and are generated sequentially, in a manner strikingly similar to the in vivo situation. Most importantly, when grafted into neonatal mouse brain, they can connect with the rest of the brain like genuine cortical projection neurons (Gaspard et al., 2008, 2009).

This model of in vitro “corticopoiesis” recapitulates all milestones of cortical development observed in vivo, including regional and temporal patterning, and therefore constitutes an attractive and robust system, which we currently use for the genetic dissection of the mechanisms of cortical neuron specification. We have started to implement a gain of function screen by overexpression of transcription factors that can later the identity of the generated neurons. We thus identified several candidates (including zBTB20, Bcl6 and Tbr2) for which we have started to define transcriptional targets through microarray and CHIP analyses. In parallel, we have started to explore the relevance of our model for cell replacement following

cortical lesions in the adult, using a combination of anatomy, physiology and functional imaging. Our first sets of data already indicate that Es-derived cortical neurons can efficiently integrate in lesioned adult cortex, with signficant and specific axonal outgrowth to cortical and subcortical targets in striatum, forebarin, midrbain and hindbrain. On the other hand have succesfully started to to implement the system to human ES cells. Using a similar default prototol, we have been able to generate forebrain progenitors and cortical neurons from hES cells, following a temporal sequence similar to the in vivo situation (Espuny et al., unpublished data). The ability to differentiate in vitro cortical neurons from hES cells would constitute a primary tool to study human cortical neuron development. Finally we have started to generate novel models of neurodevelopmental diseases, by generating specific iPS cell lines from patients displaying some of these rare diseases (Takahashi and Yamanaka, 2006). We have obtained the first candidate iPS cell lines (Hasche et al., unpublished data), which are now characterized in depth in vitro and in vivo as we have done previously for hES cells (Deleu et al., 2009).

2. Multiple roles for ephrin/Eph guidance genes in the development of the forebrain.

We previously demonstrated that ephrin/Eph genes are involved in several aspects of the development of the connectivity of the forebrain, including the patterning of cortical sensory areas and development of area-specific thalamo-cortical projections (Vanderhaeghen and Polleux, 2004; Dufour et al., 2003; Seibt et al., 2003; Egea et al., 2005; Dufour et al., 2006). In parallel we also showed an important role for ephrins in the control of forebrain 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. We have now pursued these findings by looking at the potential involvement of ephrin/Eph genes in the neuronal migration in the cortex.

To gain insight into the mechanisms involved in these processes, we have set up in utero electroporation to dissect the molecular and cellular mechanisms that control the migration of distinct populations of neurons to dorsal vs ventral domains of the telencephalon. Using these in vitro assays, we have identified several candidate guidance factors, including ephrins-B1-2, in the patterning of the migratory streams in the basal forebrain and cortex (Dimidschstein and PV, unpublished data). We follow up on these observations using 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 and striatal function in mature animals; preliminary evidence indicates that the layering pattern is abnormal in these mice, particularly in the hippocampal cortex.

3. 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.

We previoudly showed that HAR1 (*Human accelerated Region 1*), a novel non coding RNA gene that is highly conserved throughout amniotes but contains among the most highly divergent sequences in the human lineage, is strongly expressed in the human embryonic neocortex (Pollard et al., 2006). Given its potential involvement in the development and evolution of the cerebral cortex, we study the function of HAR1 in the mouse brain. To this end we are undertaking a gain-of-function approach, using electroporation of human and mouse HAR1 expression constructs, as well as a knock-in line where human HAR1 is conditionally expressed in the cortex, for which the first mice are now being analyzed, with special emphasis on potential impact on the reelin pathway. In parallel we have generated knock-out mice for the mouse HAR1 gene for which the firstt mice are now available.

Finally, we recently completed a microarray analysis that led to the identification of several hundreds of candidate genes differentially expressed between a subset of presumptive cortical areas in the human fetal cortex, using a novel approach combining three-dimensiona reconstruction of sectioned tissue (Lambot et al., 2009). Most strikingly we identified a small (around 50) subset of genes that display differential expression between presumptive language and association areas of the developing cortex in humans, which also display strong evidence of aceelerated evolution of their promoter regions in the human lineage (Lambert et al., in preparation).

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Progress report of the research group of

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Combined fMRI –intracranial microstimulation: effects on contrast response function and fMRI stimulus sensitivity

Leeland B. Ekstrom,
Pieter Roelfsema,
John Arsenault,
Wim Vanduffel

Large-scale causal functional interactions between cortical areas: from anatomy to neuro-pharmacology.

1. Contrast response function

In 2009, we published the follow-up study of our Science paper (Ekstrom et al. 2008) in which we combined intracranial microstimulation and fMRI (EM-fMRI) in awake behaving monkeys. In this second study (Ekstrom et al., J. Neurosci. 2009), we investigated the effect of FEF microstimulation on the contrast response function throughout visual cortex. In other words, we examined FEF-EM induced changes in the fMRI activity evoked by contrast varying visual stimuli. We found evidence for a non-proportional scaling of the contrast response function, resembling a contrast gain effect. Representations of low contrast stimuli were enhanced by stimulation of the FEF below the threshold needed to evoke saccades, while high contrast stimuli were unaffected or in some areas even suppressed.

2. Stimulus sensistivity

Given a recent finding of object selective neurons in the FEF and the well-known connectivity of the FEF to object selective visual regions, we sought to determine whether FEF output could also influence the relative fMRI sensitivity between stimulus types in higher-order visual areas. In a third EM-fMRI study, we measured the response in two awake, behaving monkeys to two very different types of stimuli (moving gratings and static monkey faces, respectively) positioned at the same retinotopic location in the visual field. Simultaneously, we applied electrical microstimulation (EM) to sites in the FEF with movement fields that were first aligned (congruent), and then unaligned (incongruent) with the stimulus locations. We found, particularly in higher order visual areas, that congruent FEF-EM shifted fMRI selectivity towards the stimulus preferred by that area, while in early areas we observed little change in selectivity. When we switched to the unaligned stimulation paradigm, we found that these shifts in selectivity were often in the opposite direction. These results indicate that the effects of increased FEF output in visual cortex depend not only on spatial location but also on the feature content of the stimulus presented.

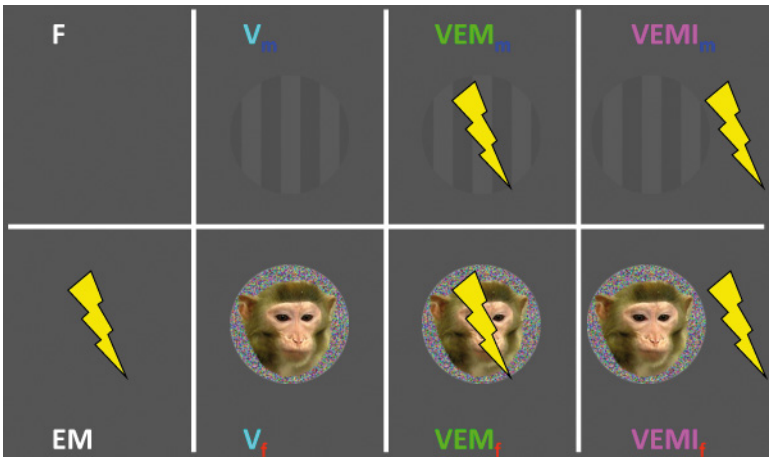

Figure 1: *Stimuli used in the third EM-fMRI experiment: We either positioned no stimuli (first column), moving gratings (first row) within the FEF-movement field or pictures of monkey faces (second row). This was either not combined with FEF stimulation (Vm, Vf), with stimulation of the matched FEF movement fields (VENm and VEMf), or a movement field was stimulated at a different position relative to that of the visual stimulus (incongruent stimulation (VEMIm, VEMIf).*

We collected cerebral blood volume (CBV)-weighted functional magnetic resonance imaging (fMRI) data from two awake monkeys (Vanduffel et al., 2001) while simultaneously stimulating the FEF (Ekstrom et al., 2008). We positioned two completely different types of stimuli at the same location within the MFs of the stimulated FEF sites: (i) low contrast moving gratings to primarily activate dorsal stream regions, and (ii) static, chromatic, high contrast face stimuli to activate more ventral stream areas. We first measured whether increased FEF output could differentially modulate stimulus selectivity in regions driven by these stimulus types. Then, using the incongruent paradigm of our previous study (Ekstrom et al., 2008), we sought to determine whether the FEF-EM effects when the stimulus and MF location are not matched are also feature-specific. In general, we found in early visual areas little and in some higher order areas profound feature-selective modulatory effects due to concurrent FEF stimulation both under the congruent and incongruent stimulation conditions.

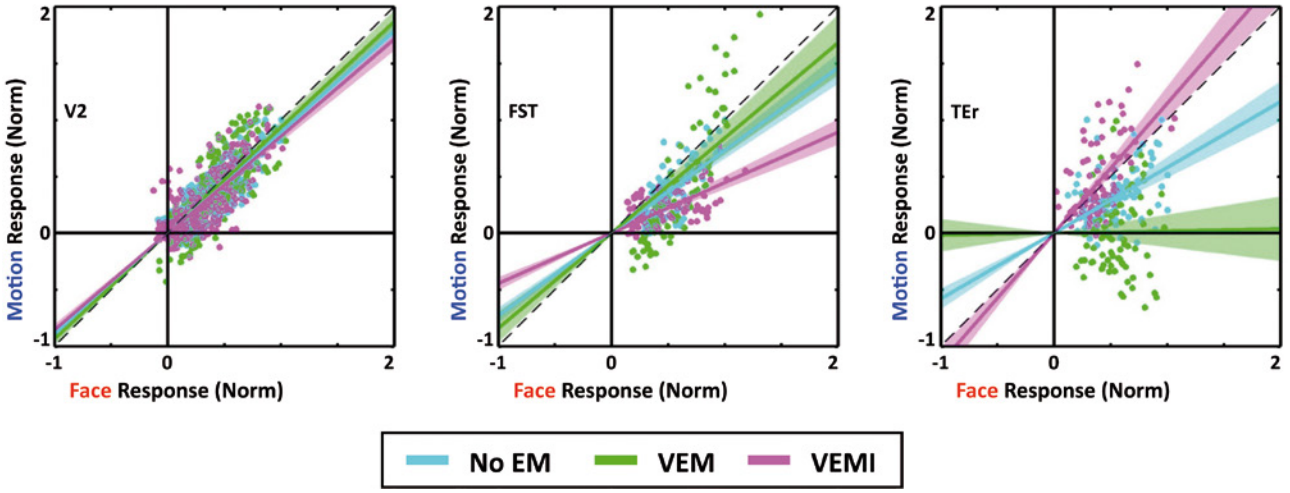

Figure 2 *Feature selectivity effects due to FEF-EM. A, Scatter plot showing for each voxel in cortical area V2 the relationship between normalized percent change in MR signal for motion (y axis) and face (x axis) stimuli during the no (cyan), congruent (green) or incongruent (purple) FEF-EM conditions. Cyan, green and pink lines are the best fit to the voxel distribution of the matching color; shaded regions indicate 95% confidence intervals for each fit, estimated by a non-parametric bootstrap algorithm. The black dashed line indicates unity slope in all panels. B and C present this same analysis for area FST (Fundus of superior temporal sulcus) and area TEr (Rostral temporal).*

For each subject, we first identified a population of visual voxels for further analysis from the fMRI contrast defined by the mean visual effect across both object classes (that is, $V_m + V_f$ versus $2 \times F$). This contrast identified voxels that were on average significantly activated by both stimuli with respect to the fixation-only condition. Using this population, we extracted the percent change in MR signal with respect to that baseline F condition for each voxel in all the other stimulus conditions (Fig. 1*A*). For each cortical area of interest, we then concatenated the population of visual voxels extracted from each subject.

In Figures 2*A-C*, we plotted the normalized percent change in MR signal in the motion (*x* axis) versus face (*y* axis) conditions for all visually-driven voxels in that particular area (cyan data points). To capture the overall behavior of the voxel population, we then calculated the best fitting line and estimated 95% confidence intervals for it using a non-parametric bootstrap algorithm. In these plots, a slope of unity would indicate that the voxels responded equally well to the *motion* and *face* stimuli. As seen in Figures 2*A-C*, the best fits to the cyan scatter plots lie below the diagonal indicating that the voxels within these three areas were better driven by *faces* compared to the low-contrast *motion* stimuli. Further, the best fitting (cyan) slopes decreased in areas FST and TEr compared to area V2, which indicates a higher sensitivity for *face* than motion stimuli in these higher-order areas while area V2 responded almost equally to both stimulus types. In essence, the TEr voxels became much less responsive to the moving grating during increased FEF output; the end result is that TEr voxels show increased discriminability between the *face* and *motion* stimuli induced by congruent FEF-EM. In area FST (Fig. 2*B*), the opposite trend is apparent though less pronounced: voxels in this area became more sensitive to the *motion* compared to the *face* stimuli. Although its confidence interval still overlapped that of the unstimulated response (cyan), the green fitted line has shifted towards the diagonal and a substantial set of voxels were clearly more *motion* than *face* selective during *congruent* FEF-EM (green voxels above diagonal).

In conclusion, we found in some higher cortical areas shifts in selectivity towards the presumably preferred stimulus in that cortical area, even though these different stimuli were presented at the same retinotopic location. When a non-overlapping MF was stimulated, we observed the opposite change – a shift away from the preferred stimulus type. Thus, we have again strengthened the observation that increased output from areas responsible for oculomotor control can modulate incoming visual activity. Here, we have shown that stimulus type, in addition to spatial location and stimulus drive, determines the modulations produced by increased FEF output. Taken together with our previous studies, this set of observations shows that presumptive feedback from higher order areas plays a crucial role in generating visual representations and in some instances may be as important as the feed-forward activation. How these modulations ultimately affect perception and behavior becomes the next, and a very exciting, question.

3. Causal relationship between FEF-EM and discrimination performance during a spatial attention task

Wim Vanduffel

In fourth study, we used FEF-EM in a spatial attention study. We trained two animals to perform an orientation discrimination task with two 3 degree diameter gratings that are presented in two different hemifields. Prior trial onset the monkeys were symbolically cued (using a colored fixation point), to attend the target grating and ignore the contralateral distractor grating. In this task, the monkeys had to

indicate with a manual response whether the attended grating was tilted clock-or anti clockwise from vertical and the orientation of the grating was adapted to teh performance levels of the monkeys using a staircase procedure. Both monkeys were able to accurately detect an orientation difference (attended grating) of less than 2-3 degrees even for gratings that were positioned at 9 degrees eccentricity.

After the monkeys mastered this task, we positioned the gratings within an FEF movement field that could be stimulated using previously implanted chronic electrodes. During the actual stimulation experiment, we first measured the just noticeable differences in orientation of the monkeys (using the staircase procedure) with the target grating positioned on the center of the FEF- movement field that would be stimulated in a subsequent series of trials. The distractor grating was positioned in a mirror position (relative to the vertical meridian) of the contralateral hemifield.

During the actual FEF stimulation experiment, the monkeys performed the same symbolically cues spatial attention task, yet the orientation of the grating was kept constant (at the JNDs defined on the same day). In the test session we either had trials in which the FEF movement field was stimulated while either the target or the distractor grating appeared in the movement field. Stimulation trials were interleaved with trials without FEF stimulation. We also manipulated the frequency of stimulation (10, 50, 200, 330 Hz) and the stimulation onset asynchrony (SOA) between EM and grating onset (EM onsets ranging between 133ms before to 133 ms after visual stimulus onset).

The preliminary behavioural results indicate that a robust decrease in reaction times can be observed when the microstimulation of the FEF preceded visual stimulus onset irrespective the frequency of stimulation. When microstimulation started at the same time as the visual stimulus or when it lagged behind, no change or a deficit could be observed. Stimulation of the FEF did not alter the JNDs of the monkeys in any of the conditions.

These behavioral results corroborate the results of Moore and colleagues who described lower contrast detection thresholds when targets were positioned within stimulated FEF movement fields.

Visual Field Map Clusters in Macaque Extrastriate Visual Cortex.

Hauke Kolster, Joseph B. Mandeville, John T. Arsenault, Leeland B. Ekstrom, Lawrence L. Wald, Wim Vanduffel

We showed, using high-resolution BOLD fMRI data in the awake monkey, that area MT/V5 and its neighbors are organized as a cluster with a common foveal representation and a circular eccentricity map. This novel view on the functional topography of area MT/V5 and satellites indicates that field map clusters are evolutionarily preserved and may be a fundamental organizational principle of the old world primate visual cortex.

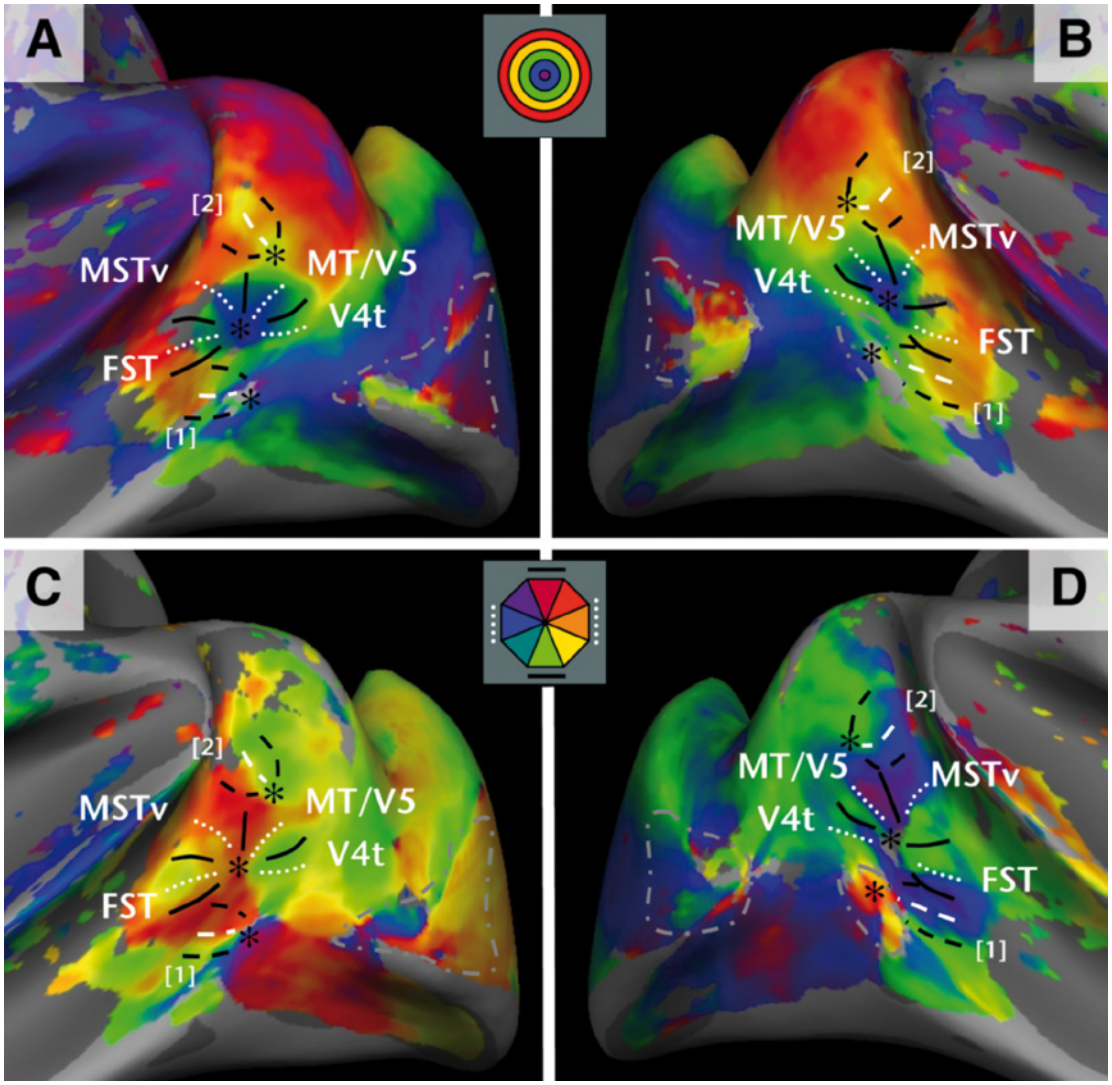

Figure 3: Retinotopy of eccentricity and polar angle representations in caudal macaque STS of subject CH. Eccentricity maps of the (A) left hemisphere and (B) right hemisphere. Polar angle maps of the (C) left hemisphere and (D) right hemisphere. The representation of central to peripheral eccentricities is coded using blue-green-yellow-red, respectively. The colors red, orange/dark blue, and green represent the upper, middle, and lower parts of the contra-lateral visual field, respectively. An asterisk indicates each representation of the centre of gaze. White dotted and black solid lines indicate horizontal and vertical meridian representations, respectively, within the MT/V5 field map cluster. The dashed white and black lines indicate horizontal and vertical meridians in field maps ventral [1] and dorsal [2] to the MT/V5 cluster. The grey dashed-dotted line indicates the central visual representation for early areas, which were not activated by the stimuli in this study. The curvature of the cortex is shown using light and dark grey to signify convexity (gyri) or concavity (sulci). IOS: Inferior Occipital sulcus; LuS: Lunate sulcus; STS: Superior Temporal sulcus; LaS: Lateral sulcus; IPS: Intraparietal sulcus; MT: Middle Temporal area; MSTv: ventral Medial Superior Temporal area; FST: Fundus of Superior Temporal sulcus area; A: anterior; P: posterior; D: dorsal; V: ventral, M: medial, L: lateral.

In two awake, fixating monkeys, we performed a phase-encoded retinotopic mapping experiment (Sereno et al., 1995). We identified a foveal representation near the fundus of the posterior portion of the STS (Fig. 3) that is anatomically distinct from the foveal representation in early visual areas (V1, V2, V3, V3A and V4). In addition, the fMRI data revealed (i) a continuous eccentricity map spanning a three quarter circle surrounding this singular foveal representation, (ii) a polar angle map showing eight alternating representations of horizontal and vertical meridians, and (iii) a field sign map indicating the existence of four individual areas joined at this foveal representation. Thus, one contralateral quarter-field plus three complete contralateral hemifield representations surrounding a common foveal representation and sharing a continuous circular eccentricity map in this portion of the STS are found consistently in all four hemispheres.

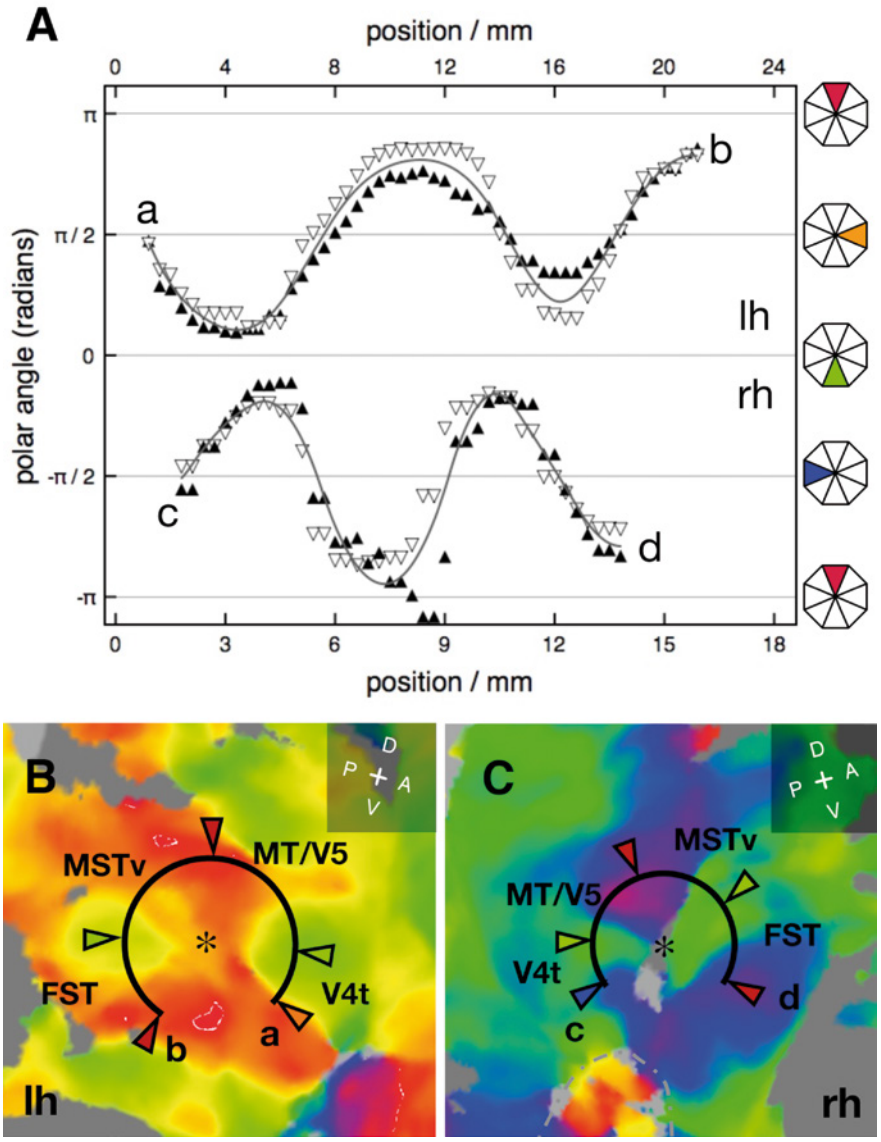

Figure 4 Polar angle phase variation within the MT/V5 cluster; subject CH. (A) Polar angle phase as a function of distance along a semi-circle within the MT/V5 cluster (CH); top: from a to b for the left hemisphere (B); bottom: from c to d for the right hemisphere (C). Open symbols (data from fig. S4D and S5D) and closed symbols (data from fig. S4F and S5F) indicate test and re-test data, respectively. Correlation between data sets: right hemisphere (bottom) $R^2 = 0.87$, $P = 1 \times 10^{-23}$ and left hemisphere (top) $R^2 = 0.71$, $P = 6 \times 10^{-12}$. The line represents a spline fit to the average of the two data sets.

Figure 4 shows the polar angle phase variation as a function of distance along a curved line surrounding the foveal MT/V5 representation based on a detailed analysis of the polar angle data. The data confirms multiple phase reversals, thereby corroborating the existence of one contralateral quarter-field and three contralateral hemifield representations. Two consecutive cycles of lower and upper-field representations can be observed if one moves along the curved lines (Figs. 4B and 2C). This topographic organization of MT/V5 and its satellites was consistently found in all four hemispheres imaged as shown by a test-retest analysis. In general, the observed functional layout resembles a pinwheel structure, exactly the hallmark of a field map cluster (Wandell et al., 2005b).

Thus, high-resolution monkey fMRI data of the posterior STS revealed three full and one half representation of the contralateral hemifield, which surround a single foveal representation and share a continuous eccentricity map spanning a three quarter circle. The functional organization that emerges

reconciles previous often contradictory maps of the STS that are mainly based on electrophysiological recordings (Komatsu and Wurtz, 1988) and anatomical tractography data (Andersen et al., 1990). The fMRI results fit surprisingly well with a recent model of cortical functional organization based on field map clusters (Wandell et al., 2005a;Wandell et al., 2007). Moreover, the present findings indicate that such clusters are not an exclusive property of the ventral stream but exist in prototypical dorsal stream regions. Exactly as predicted theoretically, we show that clusters tie together field maps known to be involved in specific perceptual functions –in this case visual motion processing. Furthermore, we show that clusters are not human-specific and are also present in old world monkeys. We conclude that visual field map clusters are evolutionarily preserved and, hence, may be a fundamental organizational principle of the Old World primate visual cortex. Therefore, we predict that improving the spatial resolution of human fMRI experiments will reveal a similar functional organization in human MT/V5+ as was observed here in the macaque.

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Coding of action categories in primate cortex.

The recognition of biological movements is extremely important for reproductive success and survival. 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, and regions of the parietal and frontal cortex are important for action recognition. We examined the analysis of dynamic action stimuli by temporal cortical neurons, including the rostral STS, of macaque monkeys.

Previously, we studied the coding by macaque temporal cortical neurons of a parameterized set of dynamic visual images of simple actions like knocking, throwing and lifting and the stimuli were rendered as stick figures. We explored mainly the dorsal and ventral bank of the rostral STS (visual areas STP and TE). The results of the single cell recordings indicated that rostral STS neurons respond selectively to temporal segments of the action movies, but not to the whole action as such. We were able to distinguish different kinds of neuronal selectivities. Firstly, neurons, mainly in the ventral bank of the rostral STS, responded as well to the action movies as to static snapshots. These neurons clearly responded to form information. Secondly, other neurons, mainly in the dorsal bank of the rostral STS, responded much less to static snapshots than to the action movies, clearly responding to motion information (**Vangeneugden, Pollick and Vogels, *Cerebral Cortex*, 2009**).

In the above described experiments we used simple arm movements in which most of the motion information was present in one point (the wrist). Now we are using more complex motion patterns, i.e. those of a walking and running human. These more complex locomotion actions have been widely used in psychophysical and functional imaging studies of biological motion perception in humans. Also, ongoing human and monkey fMRI studies in our research division use walking humans as stimuli (Jastorff and Orban, *Journal of Neuroscience*, 2009; Jastorff et al., unpublished work). The stimuli that we use are based on motion-capture data of real human subjects that were walking or running at different, controlled speeds on a treadmill. These data were obtained at ETH Zurich (collaboration with L. Van Gool). Unlike in previous neurophysiological studies of locomotion recognition, the actor does not translate across the screen in the movies, i.e. we use “treadmill” walking and running, which avoids a strong directional translation component. Importantly, our standard displays do not show a full body of a human but instead displays of cylinders connecting the joints (and a head) are used as stimuli. Despite the fact that this is an impoverished stimulus, humans perceive easily and effortless human locomotion and its direction in these displays.

This year we have completed an extensive behavioral study of the perception of such impoverished displays in monkeys. Although a vast literature exists on human biological motion perception in impoverished displays, e.g. point-light walkers, much less is known about the perception of impoverished biological motion displays in macaques. However, the latter is essential to link perception of humans and single cell response selectivities obtained in macaques. We trained 3 macaques in the discrimination of facing-direction (left versus right) and forward versus backward walking using the above discussed motion-capture-based locomotion displays in which the body features were represented by cylinder-like primitives. Discriminating forward versus backward locomotion requires motion information while the facing-direction /view task can be solved using motion and/or form. All monkeys required lengthy training to learn the forward-backward task, while the view task was learned more quickly. Once acquired, the discriminations were specific to walking and stimulus format but generalized across actors. Although the view task could be solved using form cues, there was a small impact of motion. Performance in the

forward-backward task was highly susceptible to degradations of spatio-temporal stimulus coherence and motion information. These results indicate that rhesus monkeys require extensive training in order to use the intrinsic motion cues related to forward versus backward locomotion (i.e. when no extrinsic, translatory cues are present) and imply that extrapolation of observations concerning human perception of impoverished biological motion displays onto monkey perception needs to be made cautiously. These results are presented in a detailed report which is in press in the top psychophysics journal "Journal of Vision" (**Vangeneugden et al., *J. Vision*, in press**)

We are close to finishing a single cell recording study examining the contribution of motion and form information to the selectivity for locomotion actions. We have employed the same stimuli as used in the behavioral study and recorded from two of the three trained monkeys. Unlike in previous studies from Perrett and colleagues our stimuli did not contain a translatory motion component and thus selectivity for facing direction of the walker or for forward versus backward walking cannot be due to a spatial, translation mechanism. In the case of forward-backward locomotion, it must be due to a sensitivity for snapshot sequences or motion information, while selectivity for facing direction can be based on form (posture selectivity) and/or motion information. We recorded in both dorsal and ventral banks of the rostral STS. Neurons in the ventral bank of the STS responded selectively to facing direction of the walker, but showed overall little selectivity for forward versus backward walking. A state of the art classifier (Support Vector Machines; linear kernel) was able to classify the different facing directions of the walker based on the responses of a population of 95 STS neurons with an average percent correct of > 90, while classification of forward versus backward walking was just above chance level. This shows that although ventral STS neurons can easily distinguish different facing directions they carry only a weak sequence-based signal. Further testing showed that the large majority of these neurons responded as well to the action sequences as to static presentations of the snapshots of the locomotion sequence. About half of the ventral bank STS neurons showed tuning for different snapshots and the snapshot tuning predicted the response modulations observed during the action sequences. These data suggest that the responses of ventral bank STS neurons contain only little sequence information and their contribution to the coding of actions is limited to signaling postures, as postulated for the form pathway in the Giese and Poggio model (*Nature Review Neuroscience*, 2003) of action recognition. These results have been published in abstract form (**Vangeneugden et al., *Society for Neurosciences Abstracts*, 2009**).

More recently, we have found a patch of neurons in the rostral fundus/dorsal bank of the STS that responded much less to static snapshot presentations than to actions. These motion neurons appear to distinguish forward from backward walking but this needs to be verified by quantitative data analysis and further recordings. In addition, we started a collaboration with T. Serre and T. Poggio (MIT, Boston) who are going to perform a computational modeling of our single cell data, in particular to determine the nature of the (weak) snapshot sequence signal that is present in the "snapshot " neurons.

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 to static stimuli. 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 this series of studies, we are examining the stimulus selectivity of this adaptation effect in macaque temporal cortex. We measured adaptation in macaque IT for parameterized shapes by comparing tuning for test stimuli following a brief adaptation with predictions derived from different models of adaptation. We measured

simultaneously, using the same electrode, single-cell spiking activity and local field potentials (LFPs). Adaptation was similar during two tasks: passive fixation and an attention-demanding luminance detection task. We found consistent adaptation of spiking activity and LFP power in high- (gamma) but not low-frequency bands when repeating shapes. Contrary to sharpening models of adaptation, repetition did not affect shape selectivity. The degree of similarity between adapter and test shape was a stronger determinant of adaptation than was the response to the adapter. Adaption still occurred when adapter and test stimulus did not spatially overlap, but adaptation was stronger for same, compared to different, adapters and test stimulus positions. These adaptation effects were similar for spiking and for gamma activity. In conclusion, adaptation in IT – at least when using short interstimulus intervals - is not explainable by sharpened tuning or mere firing-rate-dependent fatigue of the neuron, two mechanisms that have been proposed to underlie adaptation effects (Grill-Spector, Henson and Martin, *Trends in Cognitive Sciences*, 2006). Indeed, adaptation of IT spiking activity and LFPs in IT is strongly dependent on feature similarities in the adapter and test stimuli, in agreement with input, but not firing-rate fatigue models. These results are now in press in Cerebral Cortex (**Debaene and Vogels, *Cerebral Cortex*, in press**).

Currently we are investigating a recently proposed hypothesis that explains adaptation as resulting from the fulfillment of a perceptual expectation (or reduction in prediction error; Summerfield et al., *Nat. Neurosci*., 2008). This hypothesis is a top-down, feedback explanation of adaptation which disagrees with bottom-up mechanisms as input fatigue that we propose. The role of perceptual expectation related to adaptation is relevant for action recognition since one can predict for highly familiar actions, such as locomotion, the occurrence of a particular posture/snapshot from the sequence of previous postures.

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