Verslag – Rapport – Report

2008

G.S.K.E. – F.M.R.E. – Q.E.M.F.

www.fmre-gske.be
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Geneeskundige Stichting Koningin Elisabeth

Fondation Médicale Reine Elisabeth

Queen Elisabeth Medical Foundation

Geneeskundige Stichting Koningin Elisabeth
2008
Inleiding Verslag Activiteiten van de GSKE – FMRE

Het jaar 2008 is het eerste van de driejaarlijkse toewijzing van de onderzoekskredieten aan de onderzoeksteams geselecteerd door het Wetenschappelijk comité en bevestigd door de Raad van Bestuur werden begin 2008.
Tegen het einde van het jaar, stellen deze 16 ploegen hun evolutie rapport voor met het oog op de toekenning van de Wetenschappelijke prijzen 2009: Solvay, Baron van Gysel de Meise, CBC en Monique Brauns.

Op 29 mei heeft de overhandiging van de onderzoekskredieten en de wetenschappelijke prijzen van de G.S.K.E. aan de onderzoeksteams plaatsgevonden. Voor de eerste keer werd ook de prijs " Burggravin Valine de Spoelberch" ter waarde van 75.000 euro toegekend. De prijs werd overhandigd door mevrouw Elisabeth Speeckaert, dochter van de Burggravin Valine de Spoelberch. Familie en vrienden van de gulle schenkers hadden de gelegenheid om zich te verenigen in het Koninklijk Paleis.

Prinses Astrid, erevoorzitter van de Stichting, heeft op 26 juni, samen met een delegatie van de Stichting, een bezoek gebracht aan de laboratoria van professor Eric Bellefroid, Université Libre de Bruxelles, op de Aéropole site te Charleroi. In de herfst heeft Zij ook het laboratorium van Prof Frans Van Roy bezocht aan de Universiteit Gent.

Deze bezoeken zijn zeer belangrijk voor het imago van de Stichting en vormen voor de jonge onderzoekers een ideale gelegenheid om hun werkzaamheden aan de Prinses en de leden van de Stichting voor te stellen.
Deze bezoeken worden altijd afgerond met een receptie waar in een gemoedelijke sfeer en op een constructieve manier met wederzijds respect van gedachten wordt gewisseld tussen de Prinses en de jonge onderzoekers. De spontaniteit vormt een solide basis voor de spirit van de Stichting.

Dank zij het initiatief van het administratieve team, hoofdzakelijk van de heer Erik Dhondt, is een nieuwe drietalige folder tot stand gekomen. Deze folder stelt, buiten de website, de Stichting voor in het binnen en buitenland en creëert aldus nauwe banden.

In antwoord op een vraag van de verantwoordelijken van het ministerie van Volksgezondheid,
is de Stichting een enquête gestart over de actuele toestand van de patiënten die lijden aan Multiple Sclerose in een vergevorderd stadium en aan de ziekte van Huntington. Deze enquête werd uitgebreid gebruikt door minister Onkelinx om op basis van de resultaten een bedrag van 8 miljoen euro vrij te maken om de situatie van deze patiënten te verbeteren.
Proefprojecten zijn in januari 2009 gestart.
De samenwerking tussen het Ministerie en de Stichting werd uitvoerig voorgesteld tijdens een officiële lanceringzitting door Minister Onkelinx op 04 december, ter gelegenheid van een bezoek aan het Nationaal Multiple Sclerose Centrum te Melsbroek , in aanwezigheid van Prinses Astrid.

Uiteindelijk is op het einde van het jaar overgegaan tot de vernieuwing van een aantal leden van het Wetenschappelijk Comité van de Stichting. Het comité is samengesteld uit vertegenwoordigers van de verschillende universiteiten die benoemd worden door de respectievelijke rectoren. Deze evenwichtige en neutrale samenstelling laat toe dat het comité in alle onafhankelijkheid kan werken. De inbreng van buitenlandse deskundigen is ten gepaste tijde altijd mogelijk.

Met deze korte inleiding van het jaarlijks rapport willen we Prinses Astrid, de leden van de Raad van Bestuur en de leden van het Wetenschappelijk Comité hartelijk danken voor hun interesse en blijvende steun voor de Stichting en aan het neurowetenschappelijk onderzoek in ons land.
Zonder deze wezenlijke, materiële en morele hulp, zou het wetenschappelijk onderzoek in het domein van de neurowetenschappen in ons land nooit zo een uitstekend niveau gehaald hebben.

Prof. dr. em. Baron de Barsy
Wetenschappelijk Directeur
Brussel, 30 december 2008

Fondation Médicale Reine Elisabeth
2008
Introduction Rapport d'Activités de la FMRE - GSKE

L'année 2008 est la première des trois années d'attribution de crédits de recherche aux équipes de recherches sélectionnées par le comité scientifique et confirmées par le conseil d'administration au début 2008. Ces 16équipes présenteront en fin d'année un rapport d'évolution pour la sélection de vue en la remise des prix Scientifiques 2009 de la F.M.R.E. : Solvay, Baron van Gysel de Meise, CBC et Monique Brauns.

Le 29 mai a eu lieu la remise des crédits et des prix aux différentes équipes de recherche. Pour la première fois, la Fondation a pu attribuer le prix »Vicomtesse Valine de Spoelberch », prix bisannuel d'une valeur de 75.000 euros .Le prix a été remis par Elisabeth Speeckaert, fille de la Vicomtesse Valine de Spoelberch.et ce fut l'occasion de réunir au Palais Royal la famille et les amis des généreux donateurs.

La Princesse Astrid, présidente d'honneur de la Fondation, s'est rendue en visite, le 26 juin accompagnée d'une délégation de la fondation, au laboratoire du Professeur Eric Bellefroid, Université Libre de Bruxelles, sur le site de l'aéropole, à Charleroi. En automne, le 13 novembre Elle a visité les laboratoires du professeur Van Roy à l'Université de Gand

Ces visites fort importantes pour la visibilité et la réputation de la Fondation sont à chaque fois l'occasion pour les jeunes chercheurs de présenter leurs travaux à la Princesse et aux membres de la Fondation .Ces visites se terminent toujours par une réception au cours de laquelle des échanges entre la princesse et les jeunes se passent dans une ambiance détendue ,constructive et respectueuse, développant ainsi de façon solide, ce que nous appelons l'esprit de la Fondation « basé sur le respect ,l'échange, l'ouverture et la collaboration entre tous.

Grâce à l'initiative de l'équipe administrative, principalement de monsieur Erik Dhondt, un nouveau dépliant trilingue a vu le jour. Il permet, outre le site web , de présenter la fondation tant l'intérieur du pays qu'à l'étranger et créer ainsi de liens fructueux.

En réponse à la demande de responsables du ministère de la santé publique, la fondation a procédé à une enquête sur la situation actuelle dans notre pays des patients souffrants de sclérose en plaques très évoluées et de maladie de Huntington. Cette enquête a été largement utilisée par la ministre Onkelinx : grâce à ces données, elle a pu libérer une somme de 8 millions d'euros pour améliorer la condition de ces patients. Des projets pilotes ont débutés en janvier 2009. L'intervention conjointe du ministère et de la fondation a été largement mise en valeur lors d'une séance officielle de lancement par la Ministre Onkelinx au Centre National de la Sclérose en Plaques à Melsbroek, le 04 décembre, en présence de la Princesse Astrid ;

Enfin, en fin d'année, il a été procédé au renouvellement de certains membres du conseil scientifique de la Fondation, conseil constitué par des représentants des différentes universités et nommé par leurs recteurs respectifs. Cette composition équilibrée et neutre permet au conseil de fonctionner en toute indépendance .L'apport d'expert étranger est toujours possible si la matière le demande.

Cette courte introduction du rapport annuel est l'occasion de remercier vivement la Princesse Astrid, présidente d'Honneur, les membres du conseil d'administration et les membres du comité scientifique de l'intérêt soutenu dont ils témoignent pour l'épanouissement de la Fondation et dès lors des recherches en neurosciences dans notre pays. Sans cette aide substantielle, matérielle et morale, notre pays n'aurait pas le niveau d'excellence qu'il a actuellement dans le domaine des neurosciences.

Prof. dr. em. Baron de Barsy
Directeur Scientifique
Bruxelles, 30 décembre 2008

Geneeskundige Stichting Koningin Elisabeth
Activiteiten

2007

- **22 maart 2007:** *H.K.H. Prinses Astrid woont de inaugurele les bij van professor Guy Orban in het 'Collège de France' te Parijs.*
- **29 maart 2007:** *publicatie KCE rapport 51A - studie 2005-14 (Chronische zorgbehoeften bij personen met een niet aangeboren hersenletsel (NAH) tussen 18 en 65 jaar) in samenwerking met UZ Gent, ziekenhuis Inkendaal en VUB.*
  De studie is beschikbaar op www.kce.fgov.be (rubriek publicaties) onder referentie KCE reports vol. 51A.
- **2 mei 2007:** *bezoek van H.K.H. Prinses Astrid aan de Experimentele Onderzoeksgroep farmacologie (EFAR) van prof dr. Yvette Michotte van de Vrije Universiteit Brussel.*
- **22 mei 2007:** *uitreiking in het Koninklijk Paleis te Brussel van de*
  - *"Solvay Prize" aan prof. dr. Vincent Timmerman (Universiteit Antwerpen).*
  - *"Baron van Gysel de Meise Prijs 2007" aan prof. dr. Marc Parmentier (Université Libre de Bruxelles).*
  - *"Prijs Monique Brauns" aan prof. dr. Pierre Maquet (Université de Liège).*
- **Juli 2007:** *Studie project "Behoeften en aanbod inzake zorg en huisvesting van patiënten met Huntington en Multiple Sclerose".*
  Het in kaart brengen van de noden inzake opvang en het bestuderen van de huidige mogelijkheden en leemtes op dit vlak.
  - *Op basis van de vergelijking van deze elementen zullen een aantal beleidsaanbevelingen geformuleerd worden.*
  - *Studie in samenwerking met de heer Larmuseau David, gezondheidseconoom en vertegenwoordiger van de ministeries.*
- **26 oktober 2007:** *bezoek van H.K.H. Prinses Astrid aan de Universiteit Antwerpen, VIB-Departement Moleculaire Genetica, Onderzoeksgroep Perifere Neuropathieën bij prof. dr. Vincent Timmerman en prof. dr. Peter De Jonghe.*

2008

- **29 mei 2008:***Uitreiking in het Koninklijk Paleis te Brussel van de onderzoekskredieten G.S.K.E. 2008-2010 aan prof. P. Carmeliet (K.U.Leuven), prof. P. Janssen (K.U.Leuven), prof. W. Vanduffel (K.U.Leuven), prof. R. Vogels (K.U.Leuven), prof. V. Timmerman (UA), prof. C. Van Broeckhoven (UA), prof. A. Goffinet (UCL), prof. E. Hermans (UCL), prof. F. Van Roy (UGent), prof. M. Parmentier (ULB), prof. S.N. Schiffmann (ULB), prof. P. Vanderhaeghen (ULB), prof. P. Maquet (ULg), prof.G. Moonen (ULg), prof. L. Ris (UMH) en prof. Y. Michotte (VUB).*
  en de Wetenschappelijke Prijzen G.S.K.E. 2008:
  - *Prijs Burggravin Valine de Spoelberch aan prof. Veerle Baekelandt (K.U.Leuven),*
  - *Solvay Prize aan prof. Eric Bellefroid (ULB),*
  - *Prijs Baron van Gysel de Meise aan prof. Bruno Rossion (UCL),*
  - *Prijs Monique Brauns aan prof. Ludo Van Den Bosch (K.U.Leuven)*
- **26 juni 2008:** *Bezoek van H.K.H. Prinses Astrid aan de ULB - IBMM in Gosselies aan het laboratorium van Moleculaire Embryologie van professor Eric Bellefroid, laureaat van de G.S.K.E. - Solvay Prize 2008.*
- **13 november 2008:** *Bezoek van H.K.H. Prinses Astrid aan de UGent-VIB te Gent aan de laboratoria van de Moleculaire Biologische Cel Eenheid van het Departement voor Moleculair Biomedisch Onderzoek (DMBR) van Professor Frans Van Roy, laureaat van de onderzoekskredieten van de G.S.K.E. 2008-2010.*

Fondation Médicale Reine Elisabeth
Activités

2007

- **22 mars 2007 :** *S.A.R. la Princesse Astrid est présente à la leçon inaugurale du Professeur Guy Orban au Collège de France à Paris*
- **29 mars 2007 :** *publication rapport KCE 51B - étude 2005-14 (Besoin de soins chroniques des personnes âgées de 18 à 65 ans et atteintes de lésions cérébrales acquises) en collaboration avec UZ Gent, ziekenhuis Inkendaal et la VUB. L'étude est disponible au www.centredexpertise.fgov.be (rubrique publication) sous la référence KCE reports vol. 51B.*
- **2 mai 2007 :** *la visite de S.A.R la Princess Astrid au Groupe de Recherche Pharmacologie Expérimentale (EFAR) du prof. dr. Yvette Michotte de la Vrije Universiteit Brussel.*
- **22 mai 2007 :** *Remise au Palais Royal de Bruxelles des*
  - *prix "Solvay Prize" au prof. dr. Vincent Timmerman (Universiteit Anwerpen).*
  - *"Prix Baron van Gysel de Meise 2007" au prof. dr. Marc Parmentier (Université Libre de Bruxelles).*
  - *"Prix Monique Brauns" au prof. dr. Pierre Maquet (Université de Liège).*
- **Juillet 2007 :** *L'étude de projet "Les besoins et l'offre en matière des soins et du logement des patients souffrant de Sclérose en plaques ou la maladie de Huntington."*
  Répertorier les besoins en matière d'accueil et examiner les possibilités et les lacunes dans ce domaine.
  - *Sur base de la comparaison de ces éléments, un nombre de recommandations sera formulé aux décideurs politiques.*
  - *L'étude se fait en collaboration avec Monsieur David Larmuseau, économiste en santé.*
- **26 octobre 2007 :** *la visite de S.A.R la Princesse Astrid à l'Université d'Anvers, VIB - Département Génétic Moléculaire, Groupe de Recherche des Neuropathies périmétriques chez le prof. dr. Vincent Timmerman et le prof. dr. Peter De Jonghe.*

2008

- **29 mai 2008 :***Remise au Palais Royal de Bruxelles des prix crédits de recherche F.M.R.E. 2008-2010 aux prof. P. Carmeliet (K.U.Leuven), prof. P. Janssen (K.U.Leuven), prof. W. Vanduffel (K.U.Leuven), prof. R. Vogels (K.U.Leuven), prof. V. Timmerman (UA), prof. C. Van Broeckhoven (UA), prof. A. Goffinet (UCL), prof. E. Hermans (UCL), prof. F. Van Roy (UGent), prof. M. Parmentier (ULB), prof. S.N. Schiffmann (ULB), prof. P. Vanderhaeghen (ULB), prof. P. Maquet (ULg), prof.G. Moonen (ULg), prof. L. Ris (UMH) et prof. Y. Michotte (VUB).*
  et les Prix Scientifiques F.M.R.E. 2008 :
  - *Prix Vicomtesse Valine de Spoelberch au prof. Veerle Baekelandt (K.U.Leuven),*
  - *Solvay Prize au prof. Eric Bellefroid (ULB),*
  - *Prix Baron van Gysel de Meise au prof. Bruno Rossion (UCL),*
  - *Prix Monique Brauns au prof. Ludo Van Den Bosch (K.U.Leuven)*
- **26 juin 2008 :** *La visite de S.A.R la Princesse Astrid à l'Université Libre de Bruxelles - IBMM à Gosselies au laboratoire d'Embryologie Moléculaire du Professeur Eric Bellefroid, lauréat de la F.M.R.E. - Solvay Prize 2008.*
- **13 novembre 2008 :** *La visite de S.A.R la Princesse Astrid à l'UGent-VIB à Gand aux laboratoires de l'unité Moléculaire Biologique Cellulaire du département pour la recherche biomédicale cellulaire (DMBR) du Professeur Frans Van Roy, lauréat des crédits pour la recherche de la F.M.R.E. 2008-2010.*

Wetenschappelijke prijzen georganiseerd door de G.S.K.E.

Prix scientifiques organisés par la F.M.R.E.

Scientific awards organized by the Q.E.M.F.

2006

→ **UCB Award 2006**
Prix UCB pour la recherche scientifique en Belgique
UCB Prijs voor neurowetenschappenlijk onderzoek in België
- **Prof Dr Pierre Vanderhaeghen (ULB)**
Developmental mechanisms patterning neuronal connectivity in the cerebral cortex.

→ **Prix/Prijs Baron van Gysel de Meise**
- **Prof dr. Rufin Vogels (K.U.Leuven)**
Coding of action categories in primate cortex.

2007

→ **Prix Solvay Prijs**
- **Prof. dr. Vincent Timmerman (UA)**
Molecular genetics and biology of Charcot-Marie-Tooth neuropathies.

→ **Prix/Prijs Baron van Gysel de Meise**
- **Prof. dr. Marc Pamentier (ULB)**
Characterization of the role of G protein-coupled receptors in the central nervous system by using genetically invalidated mouse models.

→ **Prix/Prijs Monique Brauns**
- **Prof. dr. Pierre Maquet (ULg)**
Human Brain Function in sleep. Studies in man by multimodal functional neuroimaging.

2008

→ **UCB Award 2008**
Prix UCB pour la recherche scientifique en Belgique
UCB Prijs voor neurowetenschappenlijk onderzoek in België
- **Prof. Rik Vandenberghe (K.U.Leuven)**
Functional reorganisation of the language system in probable Alzheimer's disease, primary progressive aphasia, and cognitive aging.

→ **Prix Vicomtesse/Prijs Burggravin Valine de Spoelberch**
- **Prof. Veerle Baekelandt (K.U.Leuven)**
The role of the Parkinson's disease linked kinases LRRK2 and PINK1 in cellular signalling: A cellular and in vivo study.

→ **Prix Solvay Prijs**
- **Prof. Eric Bellefroid (ULB)**
Molecular mechanisms of early neural crest development.

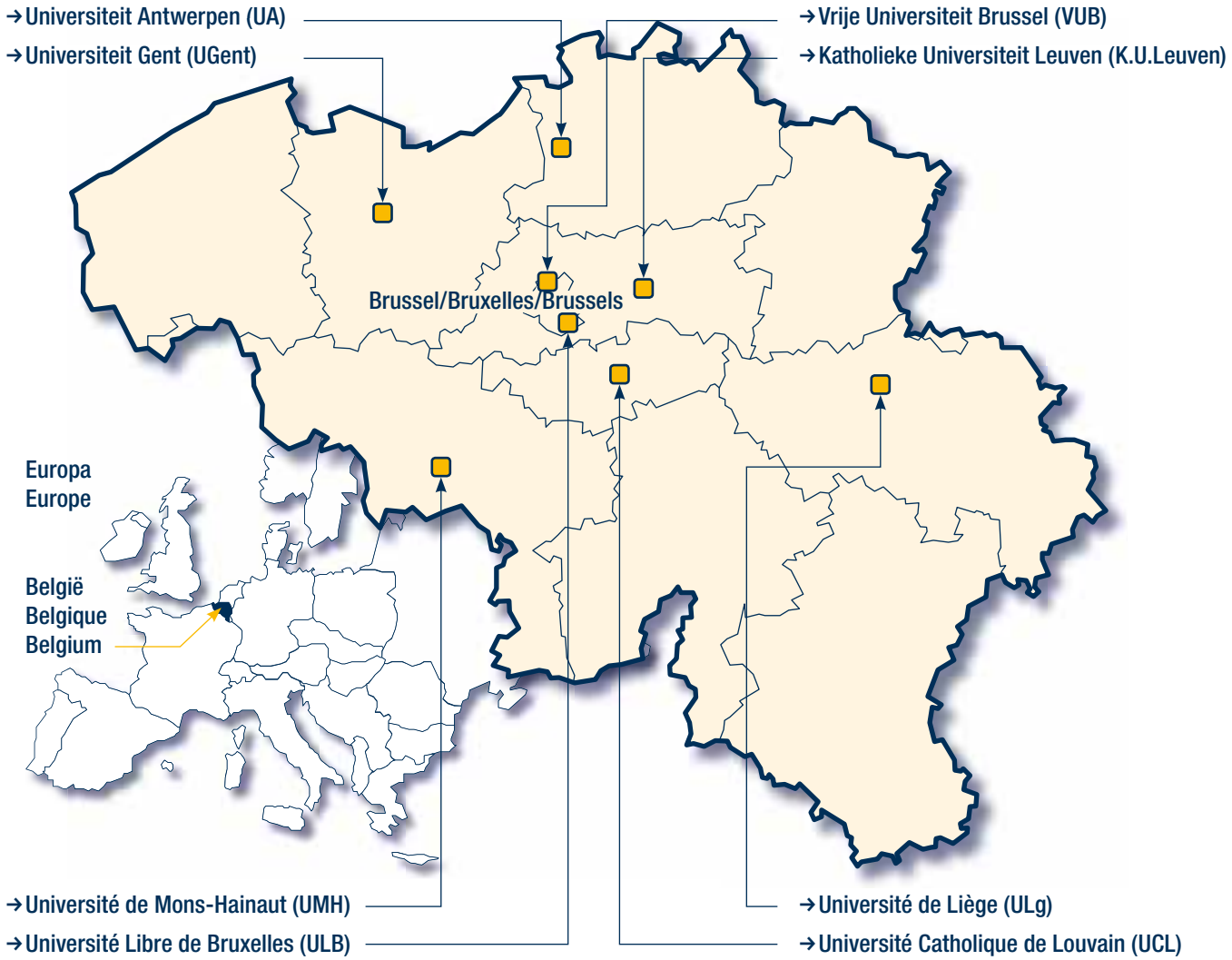
→ **Prix/Prijs Baron van Gysel de Meise**
- **Prof. Bruno Rossion (UCL)**
Clarifying the functional neuroanatomy of human face recognition by combining neuroimaging and studies of brain-damaged neurological patients.

→ **Prix/Prijs Monique Brauns**
- **Prof. Ludo Van Den Bosch (K.U.Leuven)**
Role of excitotoxicity in motor neuron degenration.

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 (2008)

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
Katholieke Universiteit Leuven

Address for Correspondence:

Vesalius Research Institute
Campus Gasthuisberg, Herestraat 49
B-3000 Leuven, Belgium
Tel No: +32-16-34.57.72
Peter.Carmeliet@med.kuleuven.be

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

Introduction:

Although originally discovered as an endothelial-specific angiogenic factor, vascular endothelial growth factor (VEGF) also has effects on neurons. Indeed, mice expressing reduced VEGF levels suffer motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis (ALS) (Oosthuyse et al., 2001). Furthermore, transgenic overexpression of VEGF or its receptor Flk1 in neurons or intracerebroventricular delivery of VEGF prolongs the survival of ALS models (Azzouz et al., 2004; Storkebaum et al., 2005). Currently a clinical trial to assess the therapeutic effect of VEGF in ALS patients is ongoing. It is thus of crucial importance to better understand the mechanisms of action of VEGF neuroprotection.

VEGF induces many of its neurovascular activities through Flk1 (Sondell et al., 2000; Oosthuyse et al., 2001; Lambrechts and Carmeliet, 2006), but also binds VEGFR1 (Flt1); the latter is also a receptor for two other VEGF family members, i.e., placental growth factor (PlGF) and VEGF-B (Olofsson et al., 1998). In contrast to VEGF, little is known about the role of VEGF-B (Nash et al., 2006). It is expressed in the heart, skeletal muscle, nervous system, and other tissues as two alternatively spliced isoforms, i.e., VEGF-B 167 and VEGF-B 186 (Olofsson et al., 1996). Mice lacking VEGF-B are viable and fertile, and exhibit minor cardiac abnormalities (Bellomo et al., 2000; Aase et al., 2001). Apart from a selective angiogenic activity in ischemic myocardium (Tirziu et al., 2007; X. Li et al., 2008), the angiogenic role of VEGF-B in other tissues and conditions remains controversial and is, overall, weaker than that of VEGF or PlGF.

The functional relevance of Flt1 has also been subject of intense debate. Flt1 binds to VEGF, PlGF, and VEGF-B with at least 10-fold higher affinity than Flk1 binds to VEGF (Shibuya, 2001). However, Flt1 has a weaker tyrosine kinase activity (Park et al., 1994), and has been proposed to act as a decoy, regulating the availability of VEGF (Hiratsuka et al., 1998). Other studies indicated, however, that Flt1 interacts with various signal- transducing proteins and generates signals (Luttun et al., 2002; Autiero et al., 2003). The precise role of VEGF-B and Flt1 in the nervous system is unclear. Flt1 is upregulated in astrocytes after administration of VEGF (Mani et al., 2005) or after injury (Choi et al., 2007), and stimulates migration of microglial cells in vitro (Forstreuter et al., 2002). Loss of VEGF-B enlarges stroke, whereas VEGF-B stimulates proliferation of neuronal cultures in vitro (Sun et al., 2004) and neurogenesis in vivo (Sun et al., 2006), and is protective in injury models, such as axotomy- and NMDA-induced cell death in the retina (Y. Li et al., 2008). VEGF-B has been presumed to exert direct neuroprotective effects, but this evidence was based on anti-apoptotic effects on smooth muscle cells and retinal pericyte cell lines (Y. Li et al., 2008). However, evidence that VEGF-B has direct neuroprotective effects on primary neurons has not been provided yet, nor has long-term delivery been shown to improve the disease course or outcome. We therefore investigated the role of VEGF-B and Flt1 in motor neuron degeneration in rodent models of ALS.

The specific aims of this proposal were to investigate

1. The role of VEGF-B and PlGF, two specific VEGF homologues binding to the Flt1 receptor, in amyotrophic lateral sclerosis ALS);

2. The mechanisms by which Flt1 modifies motoneuron degeneration in ALS;

3. The therapeutic potential of VEGF-B in ALS by gene therapy and recombinant protein therapy.

Aim 1: For VEGF-B to regulate motor neuron homeostasis, it should be expressed in the ventral horn of the spinal cord. We therefore determined its expression in wild type (WT) mice. RT-PCR analysis revealed that the VEGF-B167 and VEGFB-186 isoforms were detectable in a spinal motor neuron-enriched neuronal pellet isolated from E14 WT mouse embryos and from adult mice. Double immunohistochemistry for VEGF-B and the neuronal marker NeuN revealed that VEGF-B was detectable in large neurons, that, because of their large size and position in the ventral horn of the spinal cord were considered to be motor neurons. We also considered the possibility that VEGF-B might be differentially regulated in disease, and therefore analyzed its expression in mice expressing a SOD1G93A (hereafter referred to as SOD1) transgene. Double-immunohistochemistry for VEGF-B and NeuN confirmed that VEGF-B was detectable in motor neurons in this disease model as well. VEGF-B was not detectable in quiescent glial cells in presymptomatic SOD1 mice, but its expression became prominent and progressively upregulated in activated glial cells from symptomatic SOD1 mice. RT-PCR analysis confirmed that both VEGF-B isoforms were also measurable in primary astrocyte cultures isolated from E14 mouse embryos. Quantification of VEGF-B expression by RT-PCR in lumbar spinal cords of SOD1 mice at different stages of the disease revealed that, compared to pre-onset (day 50), VEGF-B transcript levels were increased two- to three-fold during the initial stages of the disease at day 80 and 105; this increase in VEGF-B expression is likely derived from the activated glial cells. At end-stage disease, expression of VEGF-B mRNA in lumbar spinal cords became reduced, presumably because motor neurons, that otherwise produce VEGF-B in healthy conditions, had started to disappear. Loss of motor neurons was indeed revealed by the disappearance of NF-L expression at this stage of the disease.

We also studied whether expression of VEGF-B is regulated by hypoxia or by the presence of mutant SOD1. Because of technical reasons, neuronal N2A cells stably transfected with wild-type (WT) or mutant SOD1 expression plasmids, were used, as described previously. The expression of VEGF-B in these cells was, however, not regulated by hypoxia (consistent with previous reports in other cell types), and was also not affected by the expression of WT or mutant SOD1 protein.

Aim 2: We already found that SOD1 mice lacking VEGF-B suffer from more severe motor neuron degeneration, and that VEGF-B acts as a novel neuroprotective molecule in primary motor neuron cultures. These data were already obtained prior to GSKE grant funding and were mentioned as preliminary data in the grant proposal. Since Flt1 is the receptor for VEGF-B, we also examined Flt1 expression in the spinal cord of WT and SOD1 mice. Analysis of Flt1 transcript levels by RT-PCR revealed that this receptor was expressed in a spinal motor neuron-enriched neuronal pellet isolated from WT mouseembryos or from adult mice. ELISA on the motor neuron-enriched pellet isolated from adult motor neurons confirmed that Flt1 was expressed in adult motor neurons (305±137 pg of Flt1 per mg protein; n=5). Double immunohistochemistry for NeuN and Flt1, using two different antibodies directed against the C- or N-terminal extracellular domain of Flt1, also revealed that Flt1 was detectable in spinal motor neurons of WT and pre-symptomatic SOD1 mice. In symptomatic SOD1 mice (105

days of age), Flt1 was also expressed by activated GFAP+ glial cells. As expected, immunoreactive Flt1 was also detected on blood vessels in healthy and diseased mice. To investigate whether the neuroprotective effect of VEGF-B186 was mediated through Flt1 signaling, we used mice expressing a tyrosine-kinase dead Flt1 (Flt1-TK-/- mice), which cannot transmit a biological effect of VEGF-B. We used a maximal dose of VEGF-B186 (and, for comparison, VEGF) in order to avoid that a lack of effect might be due to underdosing. As expected, survival of primary motor neurons of WT mouse embryos was stimulated by VEGF-B186 or VEGF. The percentage of surviving motor neurons after two days was 39.0 ± 3.7% in untreated cultures versus 63.8 ± 8.7% and 62.7 ± 11.1% after VEGF-B186 or VEGF, respectively (P=0.012 and P=0.034, respectively; n=6 per condition). By contrast, VEGF-B186 failed to protect embryonic Flt1-TK-/- motor neurons, while VEGF was still effective (% of surviving motor neurons: 38.6 ± 5.5% in controls versus 30.0 ± 5.6% after VEGF-B186 and 55.1 ± 8.5% after VEGF; P=0.65 and P=0.019; n=6 per condition). Thus, VEGF-B186 exerts its survival signals through Flt1, whereas VEGF is still capable of exerting neuroprotective activity through Flk1.

To further explore whether VEGF-B186 stimulated motor neuron survival directly or, indirectly, through effects on Flt1+ astrocytes, we cultured primary motor neurons of WT mouse embryos on a feeder layer of astrocytes, harvested from WT or Flt-TK-/- mice. Survival of WT motor neurons was improved by exogenous VEGF-B, regardless of the genotype of the astrocytes used, indicating that expression of Flt1 on motor neurons, but not on astrocytes, determined the survival of WT motor neurons in these co-cultures. The absolute effect of VEGF-B186 in co-cultures was somewhat smaller, which is likely due to the fact that astrocytes also release a number of other neuroprotective signals, which already provide substantial trophic support and therefore reduces the relative neuroprotective effect of exogenous neuroprotective factors. The in vivo relevance of the Flt1 tyrosine-kinase activity for motor neuron degeneration was further studied by intercrossing Flt1-TK-/- mice with SOD1 mice. SOD1xFlt1-TK+/- and SOD1xFlt1-TK+/+ genotypes developed muscle weakness at the same age (they were able to stay on a rotarod until 117 ± 3 and 118 ± 3 days of age, respectively; n=6-10; P=ns). When analyzed as single group, SOD1xFlt1-TK+/- and SOD1xFlt1-TK+/+ mice stayed on the rotarod until 117 ± 3 days and died after 143 ± 2 days of age. In contrast, SOD1xFlt1-TK-/- mice performed significantly worse: they were unable to stay on the rotarod beyond 108 ± 2 days (n=17-16; P=0.021), and also tended to survive for shorter periods (they died at 137 ± 2 days; n=17-16; P=0.071). Consistent with the analysis in SOD1xVEGF-B-/- mice, loss of Flt1 signaling had a greater effect on delaying disease onset (rotarod performance) than prolonging overall survival (see below for further discussion).

Aim 3: To explore whether the neuroprotective effects of VEGF-B could be exploited therapeutically, we delivered recombinant VEGF-B protein intracerebroventricularly (ICV) to SOD1 rats, using similar techniques that we previously used to continuously deliver recombinant VEGF (Storkebaum et al., 2005). Compared to SOD1 rats receiving control artificial cerebrospinal fluid (CSF), SOD1 rats treated with VEGF-B186 tended to fall from the rotarod 11 days later than the CSF-treated rats (131 ± 4 days for CSF-treated rats versus 142 ± 4 days for VEGF-B186-treated rats; n=11-13; P=0.073). VEGF-B186-treated rats survived significantly longer (137 ± 5 days for CSF-treated rats versus 152 ± 3 days for VEGF-B186-treated rats; P=0.016). When counting the number of motor neurons in the facial nucleus on Nissl-stained brainstem sections of pre-terminal SOD1 rats, VEGF-B186-treated rats still contained a larger number of motor neurons than CSF-treated control rats: 3,352 ± 73 motor neurons per facial nucleus after VEGF-B186 versus 2,915 ± 74 after CSF; n=8-6; P=0.029. In addition, VEGF-B186 treatment changed the disease subtype from a severe to a milder form, as was observed for VEGF (Storkebaum et al., 2005). Indeed, after VEGF-B186 delivery, significantly fewer SOD1 rats suffered forelimb disease than after control CSF (3 out of 11 VEGF-B186-treated rats versus 7 out of 10 CSF-

treated rats developed forelimb onset; P=0.047). This suggests that especially motor neurons in the brainstem and cervical spinal cord were protected, presumably because ICV delivery of VEGF-B186 results in a spatial gradient, with higher levels of VEGF-B186 in the rostral than caudal spinal cord, similar to what has been observed after ICV delivery of VEGF. When administered at low quantities (0.02 µg/kg/day ICV), VEGF was neuroprotective without causing adverse vascular effects. In contrast, ICV delivery of higher amounts (0.2 or 2 µg/kg/day) of VEGF induced angiogenesis, edema and ventriculomegaly. Since VEGF-B is a much weaker angiogenic and permeability factor, and in fact may only have some cardio-selective angiogenic activity in the ischemic myocardium, it might have a greater safety profile than VEGF. We therefore compared the effects of a similar dose of VEGF and VEGF-B186 (0.2 µg/kg/day VEGF-B186 was therapeutic) on astrogliosis and angiogenesis at one week after delivery; this analysis was performed in the brain parenchyma around the left lateral ventricle, close to where the needle was positioned (ipsilateral site), and at a site more distant from the needle implantation (i.e., around the right lateral ventricle; contralateral site).

Angiogenesis and astrogliosis were scored by immunostaining for blood vessel markers (glucose-transporter-1 or Glut1) and glial markers (GFAP), respectively. Delivery of 0.2 µg/kg/day VEGF induced angiogenesis, both at the ipsilateral and contralateral site, with some effects on astrogliosis. Remarkably, VEGF induced a substantial decrease in immunoreactivity for EBA, a marker, which only stains vessels with an intact blood-brain barrier (BBB), indicating that this dose of VEGF also increased vessel permeability ($P<0.05$ versus CSF and VEGF-B186; Table 1). By contrast, delivery of 0.2 µg/kg/day VEGF-B186 (which was therapeutic; see above) did not induce any of such angiogenic or astrogliosis side effects.

Conclusion: Our findings reveal that the VEGF-B 186 isoform plays an important role in the nervous system. The finding that VEGF-B 186 exerts neuroprotective effects on cultured neurons in vitro and in a murine model of neurodegeneration is therefore novel. In addition, our findings may also be relevant from a therapeutic perspective. Indeed, delivery of recombinant neuroprotective factors for the treatment of neurodegenerative disease has been hampered by the limited capacity of these factors to cross the BBB, or to diffuse from the CSF into the neural parenchyma. We therefore anticipate that VEGF-B 167, which binds to heparin sulfate proteoglycans and is partly sequestered in the extracellular matrix (Olofsson et al., 1996), would diffuse less after intracerebroventricular delivery and might have smaller therapeutic effects, compared with the more soluble and diffusible VEGF-B 186, on which we have focused here. Overall, delivery of VEGF-B may offer therapeutic opportunities for neurodegenerative diseases that develop spontaneously.

These data have very recently been published in the Journal of Neuroscience (see below).

Experiments planned for the upcoming year:

For the following year, we wish to perform the following experiments:

i) We will test the therapeutic effect of viral vectors expressing VEGF-B186 (AAV2-VEGF-B186) in SOD1 mice. These AAV2 vectors will be injected intramuscularly and the effect on rotarod erformance and survival will be assessed. We will analyse 15 mice per group.

ii) We have obtained some initial data indicating that VEGF-B has also a neuroprotective effect on primary sensory neurons. Mice lacking VEGF-B are further also more susceptible to a taxol-induced distal axonopathy. We would now like to assess whether these effects of VEGF-B in the sensory nervous are therapeutically relevant, and are also mediated through the Flt1 receptor.

iii) The placental growth factor or PlGF is a homologue of VEGF-B and also binds to the VEGF receptor Flt1. We will also assess the expression profile, the in vitro neuroprotective effects of PlGF, as well as the in vivo role of PlGF by intercrossing mice lacking and/or overexpressing PlGF with SOD1 transgenic mice. In addition, we will assess the therapeutic potential of PlGF.

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Progress Report of the Research Group of

Prof. dr. A. Goffinet

Université Catholique de Louvain (UCL)

Principal investigator:

André M. GOFFINET, MD, PhD

Co-investigators:

Fadel TISSIR, Chercheur qualifié FNRS
Aurélia RAVNI, PhD, Chercheur postdoc
Libing ZHOU, PhD Chercheur postdoc
Naima CHAHMI, PhD student
Yibo QU, PhD student, boursier FRIA

Developmental Neurobiology Unit:

Univ. Louvain Med. School
73, Av. E. Mounier, box DENE 73.82
B1200 Brussels, Belgium
T: +32 – (0)2764 7386
F: +32 – (0)2764 7485
Email: Andre.Goffinet@uclouvain.be
Website: http://www.md.ucl.ac.be/dene/

Genetic, molecular and cellular mechanisms of cortical development.

Background

In 2008, work supported by the FMRE in our laboratory has been focused on the role of the seven pass cadherins Celsr1, Celsr2 and Celsr3, and of the oncogene p73 during brain development. We have also initiated studies on the role of Lrrn1-3 during development.

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., 2002b; Tissir et al., 2002a). Celsr1 is expressed in zones of neural precursor proliferation and plays probably a role in the neuroepithelium, possibly by controlling planar polarity. Celsr2 is expressed in both the neuroepithelium and postmigratory neurons, and remains expressed in the adult brain. Celsr3 expression is restricted to postmigratory neurons, and is particularly high in the forebrain, including the CP. Mice with defective Celsr3 have profuse anomalies of brain wiring, a phenotype identical to that generated by inactivation of frizzled-3 (Fzd3) ((Tissir et al., 2005).

To understand better the role of Celsr3 during forebrain wiring, we used our conditional ("floxed") mutant mouse - in which the region deleted in the Celsr3 -/- mice is flanked with loxP sites - in crosses with mice that express the Cre recombinase in forebrain (Foxg1-Cre), in cortical structures (Emx1-Cre), in basal forebrain (Dlx5/6, Gsh2-Cre and Nkx2.1-Cre) and in dorsal thalamus (Ror1-Cre). This mutant allowed us to perform a genetic dissection of the role of Celsr3 during development of the anterior commissure, of reciprocal thalamocortical fibers, and of subcerebral, particularly the corticospinal tract. The data allow us to propose that Celsr3 is required intrinsically in the neurons of origin of these tracts but also in guidepost cells that guide axons during their progression. Our observations also argue strongly in favor of the "handshake" hypothesis to explain some aspects of thalamocortical wiring. These studies have been presented by L. Zhou as part of his PhD thesis, and have been published (Zhou et al., 2007; Zhou et al., 2008).

We have also studied a novel constitutive Celsr2 mutant allele. Mutant mice do survive and most of them are fertile. The phenotype is complex. First, Celsr2 -/- mice develop progressive hydrocephalus. This hydrocephalus is exacerbated in animals that are also mutated for Celsr3 in cortical (Emx1-Cre). We attribute this to defective planar cell polarity in the ependyma and have studied cilia with scanning EM as well as transmission EM and in vitro systems to analyze cilia beats. That study should be finished beginning 2009. Celsr2 -/- mice have also atrophy of dendrites in cortex, septum, hippocampus and basolateral amygdala, with some neuronal death in the same structures. We are now analyzing this in detail.

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; they are small, many have a looptail trait and most have ataxix behaviour that we still cannot explain. Some animals survive to adults but are infertile. In addition some homozygotes have a complex skin phenotype, with hair whorls 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. Studies of Celsr1 mutants have been initiated beginning 2008, and are being carried out mainly by Dr Ravni.

We have studied in some detail the migration of facial motoneurons in the different 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. This analysis is done by Yibo QU and studies of facial motoneuron migration is the main subject of her PhD project.

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 mutant mice in which the DNp73 isoform is selectively inactivated, leaving the full length p73 fully active, and in which the Cre and EGFP sequences are knocked in the p73 locus. The production of these animals was difficult to achieve, but they are now available and the mutation is fully validated. We have shown that expression of full length p73 is preserved and that both the Cre and EGFP genes are expressed as predicted. This allowed us to show that DNp73 is expressed only in a subset of Cajal-Retzius neurons that originate from the paleoventricle area, whereas the others originate from the cortical hem. We 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. We have also tested whether DNp73 acts as an oncogene in vivo (by opposing p53 and TAp73), by injecting wildtype and DNp73 -/- mice with methylcholanthrene. This study has just been completed and remains to be analyzed. We hope we can complete that first study of DNp73 mutant mice in 2009.

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). We have decided to inactivate those three genes in mice using homologous recombination. Thus far, we have produced conditional and constitutive mutants for Lrrn1 and Lrrn3, and have recently obtained an ES cell line with a floxed Lrrn2 allele. Those ES cells will soon be injected in blastocysts to generate chimeric mice. We have recently obtained the first Lrrn1 -/- and Lrrn3 -/- animals and they both survive and look healthy, which is hardly surprising given the similarity of all three proteins and the large overlap of expression patterns. We have not yet examined the brains of those mice, but have initiated production of double Lrrn1 & 3 mutants. Studies of that gene family are the thesis project of Naima Chahmi who recently joined the laboratory. This project is not expected to lead to a publication before 2010.

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Progress Report of the Research Group of

Dr. E. Hermans

Université Catholique de Louvain (UCL)

Dr. E. Hermans

Laboratoire de Pharmacologie Expérimentale,
Université Catholique de Louvain (UCL 54.10)
Avenue Hippocrate 54
B-1200 Brussels
emmanuel.hermans@uclouvain.be
Tel : 02 764 93 39

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

Neuron-glia interactions are fundamental for the physiological functioning of the nervous tissue. However, as it is likely to be the case for several diseases, the involvement of neuronal-glial crosstalks in the pathogenesis of amyotrophic lateral sclerosis has been evidenced in several studies related to the sporadic forms of the disease as well as using the transgenic models. In this context, the present project aims at further characterizing the influence of inflammatory responses on the neuroprotective/ neurorepair processes with a focus on astrocytic activities and stem cells differentiation and recruitment. With the support of the Foundation, the following projects have progressed during the last 12 months:

Chimerisation of astroglial population in the lumbar spinal cord after mesenchymal stem cells transplantation prolongs survival in a rat model of amyotrophic lateral sclerosis

(In press study in Journal of Neuroscience Research by Boucherie, Schäfer, Lavand'homme, Maloteaux and Hermans)

The potential benefit of stem cell therapies in the treatment of motor neuron diseases has been highlighted in several recent publications. Indeed, in ALS models, highly convincing are those studies showing that intraspinal injections of neural stem cells delayed the onset and prolonged animal survival through a neuroprotective potential. Exciting data suggest a non-autonomous mechanism for motor neuron death, implicating glial cells in the progression of the disease. Hence, targeting glial cells activation appears as a promising strategy to slow down ALS progression. Altogether, these studies suggest that a positive outcome could be obtained by replacing or enriching abnormal glia with healthy glial cells. With respect to accumulating data showing that mesenchymal stem cells can transdifferentiate into astrocytes when grafted into the central nervous system (Azizi et al., 1998; Kopen et al., 1999), these cells represent an attractive tool for cell therapies aiming at ameliorating the neuronal environment. As an alternative to neural stem cells, adult mesenchymal stem cells (MSCs) have been shown to exhibit neuroprotective properties when introduced into the degenerating central nervous system through different putative mechanisms including secretion of growth factors and transdifferentiation. In this project, we have injected MSCs into the cerebrospinal fluid of symptomatic hSOD1G93A rats, a transgenic animal model of familial ALS, expressing a mutated form of the human superoxide dismutase. MSCs were found to infiltrate the nervous parenchyma and migrate substantially into the ventral grey matter where motor neurons degenerate. Even though overall astrogliosis was not modified, MSCs differentiated massively into astrocytes at the site of degeneration. The intrathecal delivery of MSCs and the subsequent generation of healthy astrocytes at symptomatic stage decreased motor neuron loss in the lumbar spinal cord, preserving motor functions and extending the survival of hSOD1G93A rats. This neuroprotection was correlated with decreased inflammation, as evidenced by a lower proliferation of microglial cells and a reduction of COX-2 and NOX-2 expression. Together, these data highlight the protective capacity of adult MSC-derived astrocytes when grafted into the central nervous system and illustrate an attractive strategy to target excessive inflammation in ALS.

Cultured astrocytes derived from corpus callosum or cortical grey matter show distinct glutamate handling properties

(In press study in Journal of Neurochemistry by Goursaud, Kozlova, Maloteaux and Hermans)

Throughout the brain and the spinal cord, the grey matter is characterised by a high density of neuronal cells connected by dense networks of dendrites and short non-myelinated axons. White matter is mainly composed of longer myelinated axons that ensure communication between clusters of grey matter. As white matter is largely devoid of typical postsynaptic targets, the local release of excitatory or inhibitory transmitters has received limited consideration. However, recent neurochemical studies focusing on the corpus callosum, a white matter bundle supporting inter-hemispheric communication, have revealed a substantial vesicular release of glutamate from unmyelinated axons. Possibly contributing to some excessive activation of glutamate receptors present in glial cells and neurons, this vesicular release along axonal tracts is thought to participate in the development of excitotoxic damage associated with white matter pathologies. Accumulating data from the literature indicate that the regulation of glutamate transporters and/or mGlu receptors is critical for the modulation of glial function during excitotoxic insults and raise questions regarding the reactivity of astrocytes present in white matter. Therefore, we have compared the expression and activity of glutamate transporters, glutamine synthetase and the mGlu5 receptor in primary cultures of astrocytes derived from corpus callosum or cortical tissues of newborn rats. These populations of astrocytes showed clearly distinct phenotypes, adopting stellate or protoplasmic morphologies, respectively. In addition, white matter astrocytes showed high densities of the intermediate filament proteins GFAP, vimentin and nestin. The glutamate transporters GLAST and GLT-1, as well as glutamine synthetase, were found to be expressed at higher levels in white matter compared to grey matter astrocytes. Consistent with this aspartate uptake capacity was 3-4 fold higher in white matter cells, and the use of specific inhibitors revealed a substantial activity of GLT-1, contrasting with grey matter cells where this transporter appeared poorly functional. In addition, expression of type 5 metabotropic glutamate receptors was considerably higher in white matter astrocytes where the agonist DHPG triggered a large release of intracellular calcium. Differences in these astrocyte cultures were also observed when exposed to experimental conditions that trigger glial activation. This study highlights typical features of cultured astrocytes derived from white matter tissues, which appear constitutively adapted to handle excitotoxic insults. Moreover, the expression and activity of the astroglial components involved in the control of glutamatergic transmission are reinforced when these cells are maintained under conditions mimicking a gliotic environment.

Selective up-regulation of GLT-1 in cultured astrocytes exposed to soluble mediators released by activated microglia

(In press study in Neurochemistry International by Tilleux, Goursaud and Hermans)

During the last decades, glial cells have 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

Impaired glial glutamate uptake is commonly involved in neuronal damages observed in acute and chronic nervous disorders. As nervous insults are frequently associated with local inflammation involving microglia, this study aims at exploring the link between activated-microglia and altered glutamate uptake in astrocytes. The influence of inflammation on the regulation of GLT-1, was studied by exposing cultured astrocytes to conditioned medium from lipopolysaccharide (LPS)-activated microglia or to the key inflammatory cytokine tumor necrosis factor-alpha (TNF-alpha). Both the expression of GLT-1 and the substrate uptake activity ensured by this transporter were studied in astrocytes grown in the absence or in the presence of dBcAMP. Significant increases in GLT-1 mRNA expression and dihydrokainate sensitive uptake of aspartate were observed after 72 h of treatment. These effects were reproduced by direct exposure of the astrocyte cultures to tumor necrosis factor alpha, a major cytokine released by activated microglia. The regulation of GLT-1 activity in response to inflammatory stimuli was also evidenced in cells exposed to dibutyryl cAMP, recognised as a model of reactive astrocytes in which the expression of this glutamate transporter is constitutively enhanced. Taken together, these results suggest that the GLT-1-dependent control of glutamate neurotransmission by either naive or chemically activated astrocytes is influenced by microglia-mediated inflammation.

Glutamate-induced glioma cells proliferation is prevented by functional expression of the glutamate transporter GLT-1

(In press study in FEBS Letters by Vanhoutte and Hermans)

An efficient glial uptake of excitatory amino acids is essential in order to maintain low extracellular glutamate concentrations. This ensures an appropriate control of synaptic communication and prevents chronic glutamate neurotoxicity. Thus, impaired functioning of glutamate transporters results in increased extracellular glutamate, which is a common feature of several nervous disorders, including motor neuron diseases and Alzheimer's disease. Interestingly, reduced expression of glial glutamate transporters has been evidenced in human glioblastoma and in several glioma cell lines and elevated extracellular glutamate levels have been measured in animal models of glioma. Together, these data suggest that compromised glutamate transport could favour glioma progression by causing excitotoxic damage to normal brain parenchyma.

Therefore, the present study aimed at examining the consequence of overexpressing the high affinity glutamate transporter GLT-1 (orthologue of EAAT-2) in a model of rat glioma cells (C6). A tetracycline-dependent inducible system was used to achieve controlled expression of the glutamate transporter 1 (GLT-1) in C6 glioma cells. Non-induced cells show modest glutamate uptake and in the presence of L-cystine, these cells tend to release substantial amounts of glutamate. Overnight exposure to doxycycline increased D-[3H]-aspartate uptake, reaching similar capacity as observed in cultured astrocytes. Efficient clearance of exogenously applied glutamate was evidenced in these cells, even in the presence of L-cystine. The addition of glutamate (100 µM) to the medium of non-induced cells significantly increased their proliferation rate, an effect that was blocked when the expression of GLT-1 was induced. This suggests that impaired glutamate uptake capacity in glioma cells indirectly contributes to their proliferation. Further studies are in progress to evaluate the consequence of enhancing glutamate uptake on tumour progression when implanted in the central nervous of rats.

Progress Report of the Research Group of

Prof dr. P. Janssen

Katholieke Universiteit Leuven (K.U.Leuven)

Prof dr. P. Janssen

Laboratorium voor Neuro- en Psychofysiologie
Herestraat 49, bus 1021
B-3000 Leuven, Belgium
Tel.: +32 16 34 57 45
Fax: +32 16 34 59 93
E-mail: peter.janssen@med.kuleuven.be

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

We investigated to what extent AIP neurons and inferotemporal neurons functionally interact during the discrimination of disparity-defined 3D shapes. Two rhesus monkeys were trained on a 3D-shape discrimination task in which the monkeys have to make an eye movement to the right when the stimulus is a convex surface and to the left when the stimulus is concave. Task difficulty is manipulated by changing the percentage of the dots that give rise to the percept of the curved surface (between 0% and 100% disparity coherence). We then started simultaneous single-unit and local field potential recordings in AIP and IT during 3D shape discrimination in the same animals. We searched for responsive and disparity-selective neurons in both areas during passive fixation. For 64 responsive sites we computed spectral coherence, a measure of synchronization between two continuous signals. The LFP coherence in the beta-band (i.e. between 17 and 25 Hz) was transiently elevated in the prestimulus period. Significant desynchronization occurred after stimulus onset, followed by an increase in beta-band synchronization 200 ms after stimulus onset. The increase in synchronization depended significantly on the stimulus strength: we observed more synchronization for the 0% and 10-20% disparity coherence compared to the 100% disparity coherence stimulus. Synchronization was virtually absent in recordings in AIP and the upper bank of the Superior Temporal Sulcus, indicating that the observed effects were regionally-specific. We also computed the spike-field coherence between the multi-unit activity in AIP and the LFP in IT, and vice versa. Significantly more spike-field coherence was observed between the spikes in AIP and the LFP in IT, compared to the spikes in IT and the LFP in AIP. These results suggest that information is being transmitted from AIP (spikes) to IT (LFP). Finally, we also included two extra stimuli in our protocol: a black square and a white square, for which the animal had to make an eye movement to a single target. We observed less desynchronization after stimulus onset for the black square, which suggests that the effect of stimulus strenght on synchronization does not depend on task difficulty. Overall, these results constitute the first evidence for functional connectivity between the end stage areas of the dorsal and the ventral visual stream.

To further investigate the representation of 3D shape in area AIP, we tested 3D-shape selective neurons with curved stimuli in which either the boundary or the surface was curved in depth, or both. Boundary stimuli were created by decorrelating the dots on the surface (decorrelated boundary stimulus), by filling the surface entirely with uniform white (solid shape) and by deleting the surface dots (3D rim). The gradient of binocular disparity could vary along the vertical axis, along the horizontal axis or along both axes (a restricted surface measuring 5 deg and a large surface measuring 14 deg). We recorded from 47 3D-shape selective AIP neurons in two passively fixating rhesus monkeys. For the majority of the neurons a boundary curved in depth was sufficient to evoke selective responses: 29/47 neurons (62%) showed selectivity for at least one of the vertical boundary stimuli, and 36/47 neurons (77%) showed selectivity for at least one of the horizontal boundaries. A small fraction of the neurons (13%) did not show selectivity for any of the boundary stimuli in the test. Selectivity for horizontal gradients (20/47, 43%) was observed almost as frequently as selectivity for vertical gradients of disparity (31/47, 66%), but a small fraction of the neurons (6/47, 13%) showed opposite selectivity for the two directions of gradients (e.g. preferring convex for the vertical gradient and concave for the horizontal gradient). Only a small minority of the neurons (8/51, 16%) showed selectivity for a restricted surface in which no disparity was present on the boundary. Surprisingly, the latency of the neural selectivity for both restricted and large surface was 120 ms, much longer than the selectivity for the boundary stimuli (80 ms) and the original 3D shape in which both boundary and surface were curved in depth. These results suggest that the

3D shape representation in area AIP is primarily based on a selectivity for boundaries curved in depth.

It is unknow to what extent the presence of binocular disparity per se is necessary for AIP neurons. The possibility exists that AIP neurons are equally selective for 3D objects in which depth is defined by monocular depth cues such as shading, texture and perspective. Therefore we have started a project to assess the relative importance of binocular disparity and monocular depth cues in AIP. We have constructed a stimulus set consisting of pictures of real-world objects taken from two different viewing points (the two eyes of an observer). These images can be displayed in stereo mode (with all depth cues present), monocularly (removing binocular disparity) and uniform grey (in which also the monocular depth cues have been removed). The animal is trained and the recordings will start in the first half of 2009.

A previous fMRI study has suggested 3D-shape selective activation of a small part of premotor area F5. Therefore we investigated the selectivity of neurons in premotor area F5 to disparity-defined 3D shapes using single-cell recordings in one passively fixating monkey. Stimuli and metrics were exactly the same as in our previous studies in the inferior temporal cortex (IT) (Janssen et al., 2000). We recorded 141 responsive F5 neurons, 62 (44%) of which showed significant response differences between concave and convex surfaces that could not be accounted for by the monocular responses to the same stimuli. To verify that the observed selectivity was due to a coding of gradients of binocular disparity rather than differences in local disparities, we presented convex and concave surfaces at 5 positions-in-depth. Most (28/50, 56%) F5 neurons preserved their selectivity over positions-in-depth. We also determined the sensitivity of 38 F5 neurons by testing 3D-shape selective neurons with concave and convex surfaces of varying disparity amplitudes (from 1.3 to 0.03 degrees). The tuning for 3D shape was monotonic in some neurons, but tuned responses were also observed. These results provide critical single-cell evidence supporting an earlier fMRI study. Moreover, the data provide the first evidence of the coding of 3D-shape, i.e. to which aspects of curved surfaces the neurons are tuned, outside the visual cortex. We can now also chart possible differences in the representation of 3D shape between parietal area AIP, inferotemporal area TEs and premotor area F5. The recordings in a first monkey are finished, recordings in the second animal will begin in the next weeks.

Progress Report of the Research Group of

Prof. dr. P. Maquet

Université de Liège (ULg)

Pierre MAQUET

Cyclotron Research Centre - B30
University of Liège - Sart Tilman
4000 Liège
Belgium
Tel.: + 32 4 366 36 87
Fax: + 32 4 366 29 46
E-mail: pmaquet@ulg.ac.be

Steven LAUREYS

Cyclotron Research Centre - B30
University of Liège - Sart Tilman
4000 Liège
Belgium
E-mail: Steven.Laureys@ulg.ac.be

Characterization of spontaneous brain activity in unconscious participants by multimodal functional neuroimaging (EEG, fMRI, PET)

Our project aimed at characterizing the spontaneous brain activity and its impact on perception and cognition in humans, especially when the level or content of consciousness is altered.

Characterization of spontaneous brain activity during normal human sleep

In humans, sleep consists of two drastically different states : non rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. The cellular processes that generate the various NREM sleep oscillations (spindles, K-complexes, delta and slow rhythms) are described in great detail in animals. In humans, positron emission tomography (PET) allows the description of the gross functional neuroanatomy of NREM sleep. However, the poor temporal resolution of this technique does not allow the accurate characterization of the cerebral responses to the transient sleep events described above. For instance, slow wave activity is classically related to a decrease in cerebral energy metabolism and blood flow when measured by PET, whereas we were able to identify increases in activity associated with slow waves during NREM sleep :

Dang-Vu TT, Schabus M, Desseilles M, Albouy G, Boly M, Darsaud A, Gais S, Rauchs G, Sterpenich V, Vandewalle G, Carrier J, Moonen G, Balteau E, Degueldre C, Luxen A, Phillips C, Maquet P (2008) Spontaneous neural activity during human slow wave sleep. Proc Natl Acad Sci U S A 105:15160-15165.

Slow wave sleep (SWS) is associated with spontaneous brain oscillations that are thought to participate in sleep homeostasis and to support the processing of information related to the experiences of the previous awake period. At the cellular level, during SWS, a slow oscillation (<1 Hz) synchronizes firing patterns in large neuronal populations and is reflected on electroencephalography (EEG) recordings as large-amplitude, low-frequency waves. By using simultaneous EEG and event-related functional magnetic resonance imaging (fMRI), we characterized the transient changes in brain activity consistently associated with slow waves (>140 microV) and delta waves (75-140 microV) during SWS in 14 non-sleep-deprived normal human volunteers. Significant increases in activity were associated with these waves in several cortical areas, including the inferior frontal, medial prefrontal, precuneus, and posterior cingulate areas. Compared with baseline activity, slow waves are associated with significant activity in the parahippocampal gyrus, cerebellum, and brainstem, whereas delta waves are related to frontal responses. No decrease in activity was observed. This study demonstrates that SWS is not a state of brain quiescence, but rather is an active state during which brain activity is consistently synchronized to the slow oscillation in specific cerebral regions. The partial overlap between the response pattern related to SWS waves and the waking default mode network is consistent with the fascinating hypothesis that brain responses synchronized by the slow oscillation restore microwake-like activity patterns that facilitate neuronal interactions.

Characterization of induced brain activity during normal human sleep

We recently conducted an experiment assessing sound processing during NREM sleep to explore the apparent discrepancy between human and animal data concerning this issue. Recent evidence points out that cortical processing of external information still persists during sleep in humans. Cellular recordings in animals however suggest that the brain reactivity to external stimulation is inhibited during specific phasic activities of non-rapid-eye-movement (NREM) sleep, especially during sleep spindles. Using simultaneous electroencephalography (EEG) / functional magnetic resonance imaging (fMRI) in 13 non-sleep deprived normal human volunteers, the present study aims at assessing how spindles modulate the processing of auditory stimuli at the systems level in humans. Brain responses to pure tones were categorized in 3 types according to their occurrence during waking (TW), NREM sleep but outside spindles (TN), or spindles (TS). Expectedly, TW and TN activated the thalamus and the primary auditory cortex. Among TN, the primary auditory cortex was even more activated when the tone was followed by an evoked K-complex. By contrast, no significant brain activation was associated with TS. These results confirm that external information can be conveyed up to the cortical level during NREM sleep, a process which is associated with the production of evoked K-complexes. Furthermore, our findings demonstrate that spindles block the processing of sensory information during NREM sleep in humans, possibly contributing to the preservation of sleep continuity. **These results are summarized in a publication which is submitted for publication.**

Correlates of offline motorsequence processing during sleep

Sleep is also thought to take part in memory consolidation. We investigated the role of sleep in the consolidation of motor sequence memories. We first characterized the modifications of the neural correlates of motor sequence learning during the first 24 following the initial training :

Albouy G, Sterpenich V, Balteau E, Vandewalle G, Desseilles M, Dang-Vu T, Darsaud A, Ruby P, Luppi PH, Degueldre C, Peigneux P, Luxen A, Maquet P (2008) Both the hippocampus and striatum are involved in consolidation of motor sequence memory. Neuron 58:261-272.

Functional magnetic resonance imaging (fMRI) was used to investigate the cerebral correlates of motor sequence memory consolidation. Participants were scanned while training on an implicit oculomotor sequence learning task and during a single testing session taking place 30 min, 5 hr, or 24 hr later. During training, responses observed in hippocampus and striatum were linearly related to the gain in performance observed overnight, but not over the day. Responses in both structures were significantly larger at 24 hr than at 30 min or 5 hr. Additionally, the competitive interaction observed between these structures during training became cooperative overnight. These results stress the importance of both hippocampus and striatum in procedural memory consolidation. Responses in these areas during training seem to condition the overnight memory processing that is associated with a change in their functional interactions. These results show that both structures interact during motor sequence consolidation to optimize subsequent behavior.

Intrigued by these results, we checked in a follow up study whether this hippocampal activity elicited during initial training could predict the gain in performance overnight in another motor sequence learning task, the finger tapping task. We also tested the hypothesis that the initial hippocampal activity is selectively related to sleep-dependent motor sequence consolidation. Functional magnetic resonance imaging was used to specify the implication of the hippocampus in sleep-dependent consolidation of motor memories. Participants were scanned during practice of a finger tapping task or a visuo-motor adaptation task. They were retested three days later, with either sleep or total sleep deprivation during the first post-training night. During initial motor sequence learning, late hippocampal activity predicts the gain in performance observed at retest in sleepers but not in sleep-deprived subjects. In sleepers, responses increase in the hippocampus and medial prefrontal cortex whereas in sleep-deprived subjects, responses increase in the putamen and cingulate cortex. In contrast, for the adaptation task, performance was only maintained after sleep whereas sleep deprivation resulted in performance deterioration. No hippocampal responses were detected during training. These results stress the importance of hippocampal activity during initial practice for subsequent sleep-dependent consolidation of motor skills that imply the acquisition of a motor sequence. **These results are summarized in a paper submitted for publication.**

Modulating arousal : characterization of the impact of sleep homeostasis and circadian oscillation on regional brain function

Throughout the day, alertness and cognitive throughput are under the combined influence of circadian processes and homeostatic sleep pressure. We assessed the effects of these factors in two different experiments. The first one contrasted morning to evening chronotypes, the second was based on a polymorphism of the hPER3 gene.

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 is one of the first demonstration of the direct influence of the homeostatic and circadian interaction on the neural activity underpinning human behavior. **These results are summarized in a paper submitted for publication.**

We investigated the influence of homeostatic and circadian factors in another 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 are summarized in a paper submitted for publication.**

Characterization of induced brain activity in unconscious patients

In parallel to our research work on sleep, we are currently investigating spontaneous and induced brain activities in unconscious or minimally conscious patients

Boly M, Faymonville ME, Schnakers C, Peigneux P, Lambermont B, Phillips C, Lancellotti P, Luxen A, Lamy M, Moonen G, Maquet P, Laureys S (2008) Perception of pain in the minimally conscious state with PET activation: an observational study. Lancet Neurol 7:1013-1020.

Patients in a minimally conscious state (MCS) show restricted self or environment awareness but are unable to communicate consistently and reliably. Therefore, better understanding of cerebral noxious processing in these patients is of clinical, therapeutic, and ethical relevance. We studied brain activation induced by bilateral electrical stimulation of the median nerve in five patients in MCS (aged 18-74 years) compared with 15 controls (19-64 years) and 15 patients (19-75 years) in a persistent vegetative state (PVS) with (15)O-radiolabelled water PET. By way of psychophysiological interaction analysis, we also investigated the functional connectivity of the primary somatosensory cortex (S1) in patients and controls. Patients in MCS were scanned 57 (SD 33) days after admission, and patients in PVS 36 (9) days after admission. Stimulation intensities were 8.6 (SD 6.7) mA in patients in MCS, 7.4 (5.9) mA in controls, and 14.2 (8.7) mA in patients in PVS. Significant results were thresholded at p values of less than 0.05 and corrected for multiple comparisons. In patients in MCS and in controls, noxious stimulation activated the thalamus, S1, and the secondary somatosensory or insular, frontoparietal, and anterior cingulate cortices (known as the pain matrix). No area was less activated in the patients in MCS than in the controls. All areas of the cortical pain matrix showed greater activation in patients in MCS than in those in PVS. Finally, in contrast with patients in PVS, those in MCS had preserved functional connectivity between S1 and a widespread cortical network that includes the frontoparietal associative cortices. Cerebral correlates of pain processing are found in a similar network in controls and patients in MCS but are much more widespread than in patients in PVS. These findings might be objective evidence of a potential pain perception capacity in patients in MCS, which supports the idea that these patients need analgesic treatment.

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Progress Report of the Research Group of

Prof dr. Y. Michotte

Vrije Universiteit Brussel (V.U.B.)

Prof. dr. Yvette Michotte

Patrick Vanderheyden[2]
Ilse Smolders[1]
Heid Demaegdt[2]
Sophie Sarre[1]
Georges Vauquelin[2]
Yvette Michotte[1]
Tel.: + 32 2 477 47 48
E-mail: yvette.michotte@vub.ac.be

(1) Research group Experimental Pharmacology (EFAR), Department of Pharmaceutical Chemistry, Drug Analysis and Drug Information (FASC), Vrije Universiteit Brussel, Campus Jette, Laarbeeklaan 103, 1090 Brussel

(2) Department of Molecular and Biochemical Pharmacology (MBFA), Vrije Universiteit Brussel, Campus Oefenplein, Pleinlaan 2, 1050 Brussel, Belgium

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 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 (AT1 and AT2), 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 AT1 and AT2 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 identified as insulin-regulated aminopeptidase (IRAP) or cystinyl aminopeptidase (EC 3.4.11.3), also known as placental leucine aminopeptidase (P-LAP) and oxytocinase (Otase) (Albiston et al., 2001). IRAP is a type II integral membrane protein homologous to aminopeptidase N (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, and in epilepsy

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).

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 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 (see in review of Stragier et al., 2008). 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). The possible modulation of the above described neurotransmitter systems would also justify to investigate whether Ang IV (and IRAP) is able to affect behavioural parameters in animal models for depression.

II. Working hypotheses and specific aims of the project

The aim of this project it to unravel the mechanism(s) by which Ang IV exerts the above described effects. Insight in this field is of interest in the field of cognition as well as in the understanding of pathophysiological conditions such as Alzheimer's disease, epilepsy and depression. In first instance we are investigating whether IRAP is a true receptor i.e. that it is a major cellular recognition and signalling site for Ang IV in the central nervous system. With this respect the family of membrane-bound M1 metallopeptidases to which IRAP belongs is capable of forming homodimers providing the potential to elicit intracellular signalling as is described for growth factor and cytokine receptors (Shipp et al., 1993). Moreover the structurally related enzyme APN has been described to release intracellular calcium and to activate certain MAP kinases (Navarette Santos et al., 2000). Ang IV mediated intracellular signalling is also described in peripheral cell lines they include the release of intracellular calcium, activation of certain MAP kinases and NFκB signalling (Vanderheyden P.M.L., 2008). Logically the potential signalling of IRAP merits to be investigated in cell lines of neuronal origin.

Another hypothesis that is investigated relates with the enzymatic properties of IRAP. AT_4 ligands are found to be competitive inhibitors of the enzyme activity of IRAP. Moreover the major in vitro substrates of the IRAP enzyme, oxytocin, vasopressin and somatostatin, are known to play an important role in cognitive function (Kovacs et al., 1994, Matsuoka et al., 1995, Alescio-Lautier et al., 2000). It is therefore proposed that Ang IV and other AT_4 receptor ligands could mediate at least part of their physiological effects by inhibiting IRAP's enzymatic activity (Albiston et al., 2001). With this respect our research already provided indirect in vivo evidence that supports this hypothesis (Stragier et al., 2006). In this work we found that the anticonvulsant effect of Ang IV was linked to an increase of extracellular dopamine and serotonin concentration in the rat hippocampus and that these effects could be inhibited by a somatostatin receptor antagonist, suggesting the 'in vivo' accumulation of somatostatin to mediate these effects.

Finally Ang IV mediated effects via interaction with IRAP may be related to the co-localisation of IRAP with the glucose transporter GLUT4 in specialised insulin sensitive intracellular vesicles, as was extensively studied in adipocytes and myocytes (Keller et al., 1995; Ross et al., 1996; Sumitani et al., 1997). With this respect IRAP was recently shown to occur in intracellular vesicles in mouse brain neurones (Fernando et al., 2007). It has been suggested that the binding of AT_4 ligands may prolong the cell-surface localisation of IRAP as well as GLUT4 and therefore that the resulting increase of glucose uptake would be responsible for the biological effects of AT_4 ligands (Fernando et al., 2007; Stragier et al., 2008).

In the past year further progress was made in our understanding of the presence and properties of

IRAP in cell lines and brain homogenates and of the in vivo effects and mechanisms of action of Ang IV in the central nervous system.
In the next sections we provide an overview of our findings in 2008:
(i) Role of glucose in the memory enhancing effects of AT4 ligands
(ii) Evaluation of the role of AT4/IRAP in animal models for epileptic seizures
(iii) Evaluation of the role of AT4/IRAP in animal models for depression
(iv) Ex vivo characterization of enzyme activity of IRAP in mouse brain
(v) Characterisation of the ligand binding and enzymatic properties of IRAP in neuronal cell lines

III. Results obtained in 2008

III.1. Role of glucose in the memory enhancing effects of AT_4 ligands

The AT_4 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 to be elucidated. In recent in vitro experiments, it was demonstrated that Ang IV and LVV-H7 potentiate 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. Acute i.c.v. administration of 1 nmol Ang IV or 0.1 nmol LVV-H7 in 3 months-old Sprague Dawley rats enhanced spatial working memory in the plus maze spontaneous alternation task. Extracellular hippocampal glucose levels were monitored before, during and after behavioral testing using in vivo microdialysis. Extracellular hippocampal glucose levels decreased significantly to about 70% of baseline when the animals explored the plus maze, but remained constant when the animals were placed into a novel control chamber. Ang IV and LVV-H7 did not significantly alter hippocampal glucose levels compared to control animals in the plus maze or control chamber. Both peptides had no effect on hippocampal blood flow as determined by laser Doppler flowmetry, excluding that either peptide increased the hippocampal supply of glucose. **We demonstrated for the first time that Ang IV and LVV-H7 enhance spatial working memory in the plus maze spontaneous alternation task but no in vivo evidence was found for enhanced hippocampal glucose uptake or blood flow.**

III. 2. Evaluation of the role of AT_4/IRAP in animal models for epileptic seizures

In this topic we have studied whether deletion of the IRAP gene (i.e. IRAP knockout) can alter the seizure susceptibility in a pentylenetetrazol (PTZ) mouse seizure model. To assess (as control) the exploratory behavior, the locomotor activity was quantified in an open field test. No differences were seen between adult male and female wild-type (WT) and IRAP knockout mice. Moreover basal EEG monitoring specified that WT and IRAP knockout mice did not display spontaneous convulsions. Challenging these mice intravenously with PTZ resulted in an increased threshold in male IRAP knockout mice in comparison with male control mice. The PTZ thresholds were significantly higher for the myoclonic twitch, clonus without loss of reflexes and clonus with loss of reflexes. These behavioural data were confirmed by video-EEG monitoring. Female mice did not show any differences in PTZ thresholds. This study shows that male IRAP knockout mice are less sensitive to the development of generalized seizures following PTZ. **These findings show unequivocally that IRAP is involved in seizure generation.** It is obvious that further work is necessary to unravel the mechanism(s) of this modulation.

III.3. Evaluation of the role of AT_4/IRAP in animal models for depression

In this topic we have explored whether a role of IRAP in an animal model for depression could be shown. For this purpose adult male and female control and IRAP knockout mice were subjected to the tail suspension test. This behavioural test was carried out in a device that consisted of a white box (30 x 30 cm) without cover with a depth of 20 cm that was put with the bottom of the box perpendicular on the table. An iron rod with a length of 10 cm was fixed to the top side of the box and was used to suspend the mice individually with Leukotape®. Two independent researchers of which one was blinded for the treatment manually recorded the immobility time (in s) of each mouse during the 5-min period. To validate this system, mice received an i.p. injection of the antidepressant imipramine (40 mg/kg) 30 minutes before the test. As expected imipramine significantly decreased the mean immobility time as compared to the control group. However, there were no differences in immobility time between wild-type and IRAP knockout mice of both sexes. In conclusion, up to now, we cannot provide evidence for a role of IRAP in animal behaviour models for depression.

III. 4. Ex vivo characterization of enzyme activity of IRAP in mouse brain

In previous work of Stragier et al. (2006) we found that the Ang IV-mediated inhibition of pilocarpine induced seizures in rats was prevented by a somatostatin-2 receptor antagonist, suggesting the involvement of somatostatin-14. Similar results were obtained in mice (see III.x). These results suggest that AT_4 ligands may exert these effects by inhibiting the enzymatic activity of IRAP, resulting in the accumulation of substrates of this enzyme. In order to provide further evidence for this mechanism, we have characterised the enzyme activity (and its inhibition by AT_4 ligands) in ex vivo homogenates of cerebral cortex of control and IRAP knockout mice. By using the synthetic and colorigenic substrate l-leucine-p-nitroanilide we demonstrated that about **70 % of the enzyme activity could be attributed to IRAP** and that the remaining activity is caused by the closely related APN. This proportion of IRAP mediated enzyme activity was also confirmed by concentration-inhibition experiments with Ang IV, AL-11 (a recent developed IRAP ligand with improved stability when compared to Ang IV and that was obtained in collaboration with prof. D. Tourwé at the Vrije Universiteit Brussel, Lukaszuk et al, 2008) and with compound 7B (a stable non-peptide aminopeptidase N-selective inhibitor obtained in collaboration with Prof. Yiotakis, Athens).

These brain homogenates will be used to assess whether the neurotransmitter somatostatin is indeed metabolised by IRAP and if so, whether this could be inhibited by IRAP ligands. For this purpose we have set up **a radioimmune assay which enables us to detect picogram amounts of somatostatin** in the supernatans of the membrane preparations. Subsequently we will incubate exogenously applied somatostatin in the presence of homogenates of cerebral cortex from control and IRAP knockout mice. Similar experiments will be carried out with hippocampal homogenates. It cannot be excluded that IRAP ligands may also exert certain in vivo effects via the accumulation of other neuropeptide substrates of this enzyme. Therefore a similar approach will be applied to assess the ex vivo metabolism in brain homogenates of potential IRAP substrates such as vasopressin and oxytocin.

III. 5. Characterisation of the ligand binding and enzymatic properties of IRAP in neuronal cell lines

The interaction of two new radioligands ([^{3}H]Ang IV and [^{3}H]AL-13) with AT_4/IRAP was studied in a hippocampal neuronal cell line (P40H1). At first, this study examined whether differences between the use of tritiated Ang IV and iodinated Ang IV can be detected. Iodine is a large and hydrophobic atom

and its use can change the pharmacological and physicochemical properties of the natural ligand. A pharmacological profile was established using [^{3}H]Ang IV competition binding assays. **This profile showed large resemblances with the binding profile of IRAP/AT$_4$ for both type of radioligands**, as established in earlier studies using [125I]Ang IV competition binding assays in different cell lines (Demaegdt et al., 2004a; Demaegdt et al., 2006, Lew et al., 2003, Lee et al., 2003). In this study as well as in earlier studies (Demaegdt et al., 2004b, 2006), only binding to the IRAP apo-enzym was measured because of the presence of divalent cation chelators EDTA and 1,10-PHE (E/P). The binding profile established using [^{3}H]AL-11 competition binding assays in the presence of chelators revealed a similar profile to that of apo-IRAP (established using [^{3}H]Ang IV or [^{125}I]Ang IV competition binding assays). Since the use of these different radioligands revealed a similar binding profile, the choice of the radioligand in apo-IRAP binding assays can be determined by the concentration of binding sites in the cell material.

Up to now it was impossible to perform [^{125}I]Ang IV binding assays in the absence of chelators. The use of tritiated AL-11 (a stable Ang IV analogue, selective for IRAP versus the AT1 receptor) made it possible to carry out radioligand binding assays under this condition. The binding profile established using [^{3}H]AL-11 competition binding assays in absence of chelators corresponded to the enzyme profile of IRAP. Large differences could have given an indication of possible alternative binding sites. As a consequence **[^{3}H] AL-11 binding can be measured for binding to the catalytic active IRAP (in absence of chelators) and for binding to apo-IRAP (in presence of chelators).** However, depending on IRAP being in apo-enzyme form or not, differences in affinity of some ligands are found. These differences cannot solely be caused by degradation. A modification of the binding site due to the presence or absence of Zn^{2+} is an alternative explanation.

The enzyme inhibition assays in the P40H1 cell membranes pointed to the presence of two (or more) enzymes IRAP and APN. The pharmacological profile of the enzyme with high affinity for Ang IV and AL-11 and low affinity for 7B (APN selective) corresponded to the binding profile which was established using [^{3}H]AL-11 competition binding assays (-E/P). It also corresponded to the enzyme profile of IRAP in other cells. The second enzyme (i.e. with low affinity for Ang IV and AL-11 and high affinity for 7B) corresponded to APN. During the radioligand binding assays however, no high affinity binding for APN was measured neither in absence nor in presence of chelators.

IV. 2008 Publication List

MBFA

- Demaegdt, H., Smitz, L., De Backer, J.P., Le, M.T., Bauwens, M., Szemenyei, E., Tóth, G., Michotte, Y., Vanderheyden, P.M.L., Vauquelin, G. Translocation of the Insulin Regulated Aminopeptidase to the cell surface: detection by radioligand binding. Brit J Pharmacol, 2008, 154, 872-881. (SCI factor: 3.82)
- Demaegdt, H. Chapter IV: How is the Insulin Regulated Aminopeptidase involved in the physiological and in vitro effects of Angiotensin IV? Review. 'Angiotensin Research Progress', Eds: Hina Miura and Yuuto Sasaki, Nova Publishers group, pp 93-119.
- Lukaszuk, A., Demaegdt, H., Szemenyei, E., Tóth, G., Tymecka, T., Misicka, A., Karoyan, P., Vanderheyden, P.M.L., Vauquelin, G., Tourwé, D. beta-homo-amino acid scan of Angiotensin IV. J Med Chem, 2008, 51, 2291-2296. (SCI factor: 5.11)
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- Vanderheyden, P.M.L., From angiotensin IV binding site to AT4 receptor. Review. Mol. Cell. Endocrinol., in press (SCI factor 2.971)

FASC

- De Bundel, D., Smolders, I., Vanderheyden, P., Michotte, Y. Ang II and Ang IV: unraveling the mechanism of action on synaptic plasticity, memory, and epilepsy. CNS Neurosci Ther. 2008 ,14(4):315-339. (SCI impactFactor 2007= 3.795)
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Progress Report of the Research Group of

Prof. dr. G. Moonen

Université de Liège (ULg)

Prof. dr. Gustave Moonen

Service de neurologie
CHU et Université de Liège
Sart Tilman
4000 Liège
Belgium
Tel.: 32 4 366 85 55
g.moonen@ulg.ac.be
neurologie@ulg.ac.be

Laurent Nguyen

Developmental Neurobiology Unit
Center for Cellular and Molecular Neurobiology
University of Liège
4000 Liège
Tel.: 04 366 59 87
Fax: 04 366 59 12
lnguyen@ilg.ac.be
www.cncm.ulg.ac.be

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

The cerebral cortex develops from the dorsal telencephalon and contains neurons that are distributed within layers and are regionally organized into specialized areas that underlie sophisticated cognitive and perceptual abilities (Rash and Grove, 2006). Cortical lamination follows an « inside-out » sequence of neuronal placement and maturation that arises from the sequential birth and orderly migration of both, pyramidal projection neurons generated in the dorsal telencephalon (reviewed in Gupta et al., 2002) and, GABAergic interneurons born in the ganglionic eminences (GE) (Anderson et al., 1997; Tamamaki et al., 1997). 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 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 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. Thus, the identification of new cytoskeleton regulators is essential to shed more light on the molecular mechanisms responsible for the generation of fully differentiated cortical interneurons. Our current experiments are aimed at identifying key molecules that control cytoskeleton remodelling during the migration and branching of cortical interneurons.

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.

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

The molecule $p27^{Kip1}$ belongs to the Cip/Kip family of proteins that are promoting the exit of cell cycle in various tissues. We have previoulsy shown that in addition to this function, $p27^{Kip1}$ coordinates neurogenesis in the cortex by promoting the differentiation and radial migration of projection neurons. Our findings highlighted a functional cooperation between Cip/Kip and RhoGTPases during projection neuron migration whereby $p27^{Kip1}$ promotes migration by blocking RhoA activity (Nguyen et al., 2006).

Cortical GABAergic interneurons are born in the medial and caudal GE (MGE and CGE) and are using mutiple tangential routes to reach the developing cortical plate. Their migratory behaviour appears to be orchestrated by specific combinations of repulsive, attractive and motogenic cues that includes neurotransmitters (reviewed in Heng et al., 2007). Importantly, our analyses performed on embryonic

brains revealed the expression of $p27^{Kip1}$ in progenitors located in the subventricular zone of the MGE/CGE as well as postmitotic cortical interneurons. Interestingly, $p27^{Kip1}$ was expressed in both the nucleus and the cytoplasm of these neurons, suggesting that it could harbour 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 $p27^{Kip1}$. In situ hybridization experiments performed on E12 brain sections indeed showed the expression of $p57^{Kip2}$ in the GE.

While p27 was dispensable for the proliferation and birth of interneurons, we observed a reduced number of LhX6-positive interneurons in the lateral cortex of p27 knockout E14 embryos. Since no increased cell death was responsible for this apparent change, we suggested that $p27^{Kip1}$ regulates the migration of cortical interneurons. To test this hypothesis we conditionally removed p27 in Dlx5,6-positive cortical interneurons from p27lox embryos (Chien et al., 2006) (generated by Dlx5,6 Cre-IRES-GFP X p27lox breedings) and observed similar cellular defects, thus supporting a cell autonomous requirement of p27 for tangential migration. We are currently performing time lapse analyses to understand whether the migration defects result from a reduction in speed of migration or an impaired capability of interneurons to enter into the cortex. In addition, p27 likely regulates the 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 plan to unravel the molecular pathways lying downstream p27 that regulate the tangential migration of cortical interneurons. We will also assess both, the terminal branching and fate specification of interneurons that lack p27. For this we will perfomed confocal microscopy analyses of cortices from postnatal animals at P0 and P7, respectively. The branching will also be quantified on cultured interneurons that have been dissociated from the MGE of E14 embryos generated by Dlx5,6 Cre-IRES-GFP X p27lox breedings.

2. 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.

This highly conserved complex of 6 subunits (Elp1-Elp6) was identified associated with the hyperphosphorylated form of yeast RNA polymerase II ((Hawkes et al., 2002; Kim et al., 2002) and is assembled by the scaffold protein Elp1 (Close et al., 2006; Petrakis et al., 2004). Elp3, the catalytic subunit, harbours motifs found in the GNAT family of histone acetyltransferases (HATs) (Wittschieben et al., 1999) and 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 for this complex in transcript elongation. Other reports also provided evidences for a role of Elongator in exocytosis and tRNA modification in the cytoplasm (Esberg et al., 2006; Huang et al., 2005; Rahl et al., 2005). We showed that an acute disruption of its activity in dorsal progenitors results in radial migration delays and defective terminal branching of projection neurons that come with a reduction in α tubulin acetylation. Importantly, this complex interacts with the microtubule cytoskeleton where Elp3 may directly acetylate α tubulin, a postranslational modification known to regulate the intracellular trafficking that is critical for cell shape remodelling during migration and terminal branching (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 take part to the regulation of the migration and branching of cortical interneurons. For this purpose, we are currently generating a transgenic mouse line (Elp3lox) that will be used for conditional removal of Elp3 in Dlx5,6 Cre-IRES-GFP interneurons. This strategy will allow us to analyse the generation, migration and branching of Elp3 knockout cortical interneurons.

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Progress Report of the Research Group of

Prof. dr. M. Parmentier

Université Libre de Bruxelles (ULB)

Prof. dr. Marc Parmentier

Institut de Recherche Interdisciplinaire en
Biologie Humaine et Moléculaire (IRIBHM)
Faculté de Médecine
Université Libre de Bruxelles (ULB)
Campus Erasme
808 route de Lennik
B-1070 Bruxelles
Tel.: + 32 2 555 41 71
Fax: + 32 2 555 46 55
E-mail: mparment@ulb.ac.be

Catherine Ledent

Institut de Recherche Interdisciplinaire en
Biologie Humaine et Moléculaire (IRIBHM)
Faculté de Médecine
Université Libre de Bruxelles (ULB)
Campus Erasme
808 route de Lennik
B-1070 Bruxelles
Tel.: + 32 2 555 85 11
Fax: + 32 2 555 46 55
E-mail: cledent@ulb.ac.be

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

1. The glucocorticoid-induced receptor (GIR) receptors

Glucocorticoid-induced receptor (GIR or GPR83) is a receptor with predominant expression in brain and thymus. High levels of GIR expression have been described in limbic forebrain and hypothalamic regions of the brain of mouse, rat and human, suggesting a role for GIR in memory, cognition, stress, reward or the control of emotion. We have generated a knock-out model for GIR.

GIR is expressed in cholinergic interneurons of the striatum. In our targeting vector, part of the GIR coding region was replaced by a tau-lacZ fusion gene (placed under control of the natural GIR promoter) and selection cassettes. The tau-lacZ fusion protein was visualised by X-Gal staining of brain slice in heterozygous animals. We observed a strong cellular staining in olfactory bulb, olfactory tubercle, nucleus of the olfactory tract, piriform cortex and scattered cells in dorsal striatum. Few cells were also stained in the thalamus, hypothalamus and CA3 region of hippocampus. In all cases, the neuronal processes were also stained. In the olfactory bulb, the localisation and shape of processes indicated the peri-glomerular identity for the GIR expressing-neurons. In the dorsal striatum, the size of the cell bodies and the arborisation suggested that GIR was expressed in large aspiny cholinergic interneurons. Co-immunostaining revealed 89% co-localization of GIR with choline acetyl-transferase (CHAT), a marker of cholinergic neurons. However cholinergic interneurons are also located in the ventral striatum where very few GIR-positive neurons were found, indicating the existence of a subclass of cholinergic interneurons in the dorsal striatum expressing GIR. Few other GIR-positive neurons did not colocalise with CHAT and had a distinctive elongated cell shape, with a single process.

GIR invalidation decreases motor learning ability. Cholinergic interneurons of the striatum have been shown to be involved in motor learning. We assessed the locomotor ability of GIR knock-out mice in four tests probing striatal and/or cerebellar function. In the rota-rod test, designed to assess motor coordination, knock-out mice displayed a reduced aptitude for coordinated movements and did not manage to learn the test even after 5 days of conditioning. Similarly, knock-out mice were inefficient compared to wild type in the strength-grip test, a test that involves muscular strength and coordination. The runway test and the foot print test are designed to explore cerebellar deficiencies. Wild type and knock-out mice displayed similar abilities in both tests. In addition to motor control, striatum also participates to motivational control and rewarding effects of drug of abuse. Dopaminergic activity in nucleus accumbens is primarily implicated in the rewarding properties of drugs such as cocaine. We assessed the rewarding effects of cocaine in the conditioned place preference test but did not observed differences between wild type and knock-out mice.

GIR invalidation affects the activity of the striatal network. Dopaminergic and cholinergic afferences modulate the GABAergic medium spiny neurons (MSNs). MSNs are commonly divided in two major subsets on the basis of their axonal projections and on the expression of neuropeptides and dopamine receptor subtypes. Striatonigral MSNs co-express D_1 dopaminergic receptors, substance P and dynorphin, while striatopallidal MSNs co-express D_2 dopaminergic receptors, A_{2a} adrenergic receptors and enkephalin. Changes in striatal network activity are typically correlated with the expression level of these neuropeptides. Quantitative in-situ hybridization showed that enkephalin transcripts are slightly decreased while substance P and dynorphin trannscripts were significantly increased in the caudate

putamen of knock-out mice. In situ binding suggested that D1 and D2 receptors were not significantly modified in the striatum or cortex of GIR knock-out mice.

The number and distribution of cholinergic neurons in the striatum were not modified in knockout mice. We monitored the acetylcholine secretion *in vivo* in the dorsal striatum with a microdialysis probe. We observed a decrease in basal acetylcholine secretion in knock-out mice as compared to wild type mice. Scopolamine, an M2 receptor antagonist that suppresses the autocrine negative feedback of acetylcholine, increased acetylcholine secretion in both genotypes, but knock-out mice returned faster to a lower basal level. Induction of early genes such as c-fos or Zif-268 has been proposed to reflect neuronal activity. Zif-268 transcript levels were increased in the caudate putamen of knock-out mice, more particularly in the most superficial parts of the structure, where most GIR-positive cholinergic interneurons are located.

GIR invalidation affects the stress axis. Glucocorticoids and forskolin were initially described to induce GIR expression in a thymoma cell line. In the brain however, GIR expression is decreased by glucocorticoids in hypothalamus, suggesting a possible role in the regulation of stress. Plasma corticosteroid concentrations were significantly lower in knock-out mice in basal conditions, and the difference between genotypes became larger in stressful situations (LPS administration, restraint, open field test).

Motor control in stressful conditions. We monitored the spontaneous locomotor activity in actimetry boxes. On day 1, wild type and knock-out mice displayed similar locomotor activities. After habituation and learning (day 2 and 3), knock-out mice displayed a lower activity for both the horizontal and vertical components. In the open field test, which is more stressful, GIR knock-out mice displayed a significantly higher locomotor activity during the three days of the test and spent more time in the central area. These results suggest an anxiolytic-like phenotype. Mice were tested in behavioral tests probing more specifically anxiety, namely the light and dark box and the elevated plus maze. Although the knockout animals displayed higher locomotor activity in these arenas, the parameters reflecting anxiety were not significantly different between genotypes. We assessed the effect of the D1 agonist SKF38393 and the M2 antagonist scopolamine on the locomotory activity of wild type and knock-out mice in the open-field test. Both drugs resulted in a similar biphasic effect on GIR knockout mice. At low doses, they did not affect wild type mice but decreased the locomotor activity of knockout mice to the same level as wild type animals. At higher doses, they increased in parallel the activity in both genotypes. Finally, we also tested knock-out mice in tests commonly used for probing antidepressant compounds. In the tail suspension test and the forced swimming test, knock-out mice displayed a depressive-like behaviour: they spent significantly more time immobile than wild type mice. In both tests, the injection of fluoxetine, an anti-depressant compound, reduced the time spent immobile for wild type and knock-out mice.

Pharmacological activation of GIR by poly-unsaturated fatty acids and NPY peptides. Peptides of the NPY family have been described to bind and activate GIR expressed in COS-7 cells (NPY(3-36) > PYY (3-36) > NPY > Leu,Pro-NPY), suggesting that GIR is a novel NPY receptor with a Y2-like pharmacology. Independently, several poly-unsaturated fatty acids (PUFA) were reported in a patent to act on GIR, following their isolation from pig brain. Both hypotheses were tested, following the expression of human GIR (hGIR) in CHO-K1 cells also expressing G16 and apoaequorin. hGIR-overexpressing cells were specifically activated by docosahexanoic acid (DHA), 9-cis retinoic acid (9-cRA), and arachidonic acid but not NPY or PYY (3-36). A cell line expressing the Y2 receptor was activated by NPY and PYY (3-36) but not by DHA or 9-cRA, while control cell lines expressing other

receptor did not respond to these ligands. We next assessed the effect of combinations of PUFAs and Y2 agonists on hGIR-overexpressing cell. DHA in combination with PYY (3-36) or NPY induced a stronger activation. In the presence of DHA, the EC_{50} for PYY-3-36 decreased to the nanomolar range and the E_{max} was increased.

We also monitored the internalisation of GIR following activation in COS-7 cells transiently expressing hGIR or a fusion of hGIR tagged with GFP at its C-terminus. We observed the GPCR Internalisation was detected either by fluorescent confocal microscopy or FACS. DHA at 10 µM promoted internalisation of hGIR (62% at 45 min), which clustered in a peri-nuclear endosomal compartment. A control receptor similarly tagged with GFP did not internalise in the same conditions. NPY or PYY (3-36) alone did not internalise hGIR but combinations of DHA and PYY (3-36) promoted faster internalisation and at lower concentrations than DHA alone. These results suggest that Y2 agonists can activate hGIR, but only in the presence of DHA.

We tested by in situ binding assay whether GIR could bind NPY peptides in mouse brain. Using ^{125}I-PYY (3-36) as a tracer and Leu,Pro-NPY as a Y1 competitor, we could determine Y2-like binding sites in specific brain regions. We observed a significant reduction of ^{125}I-PYY (3-36) binding sites in the hippocampus and amygdala of GIR knock-out mice as compared to wild type animals. Binding in striatum was very low and we were uable to observe differences in this region. Similarly, no difference was seen in septum, where GIR is not expressed.

We also tested whether part of the physiological effects of PYY could be mediated by GIR. Following i.p. injection, PYY (3-36) decreased locomotory activity of wild type mice, but not of GIR knock-out mice, suggesting that GIR is required for the locomotory effects of PYY(3-36) in vivo. Finally we quantified NPY expression in striatum and hypothalamus of wild type and knock-out mice. We observed a significant increase in NPY expression in the striatum (but not hypothalamus) of knock-out as compared to wild type mice.

Altogether, we have shown that GIR is expressed by cholinergic interneurons of dorsal striatum, and that inactivation of this receptor is associated with cholinergic hypoactivity in the striatum (Laurent et al. In preparation). This leads to an alteration of coordinated movement and increased locomotory activity in stressful conditions. We also showed that GIR is activated in a cooperative manner by NPY peptides and polyunsaturated fatty acids, and that the receptor is involved in some of the activities of NPY peptides in vivo.

2. The GPR10/prolactin releasing factor system

Prolactin-releasing peptide (PrRP) is the ligand of the receptor GPR10. As its names indicates, PrRP was described originally as a regulator of prolactin release. However, it has since been involved in a growing number of physiological processes, among which the control of feeding behavior, pain and neuroendocrine function. We have generated a knock-out model for GPR10 and these mice were tested across a wide range of behavioral and physiological assays.

We have demonstrated an important role of the PrRP-GPR10 system in the modulation of the various actions of opiates (Laurent et al. 2005). We have also established a central role of GPR10 in the control of stress reactions, with a lower activity of the hypothalamus-pituitary-adrenal axis in knock-out mice in basal conditions and following a number of stressfull conditions. This low corticostreroid tone was associated with a spontaneous chronic inflammatory disease of the liver, leading to steatosis and fibrosis. As our animals were not kept in a SPF environment, we could not determine the exact origin of this chronic disease, and its relation to the central control of the hypothalamus-pituitary-adrenal axis. We have introduced the GPR10 knockout line in a SPF environment, and have bred them for a few generations. We are now starting the experiments that will allow to determine whether the inflammatory syndrome is spontaneous or indused by a specific pathogen, and whether it is restricted to the liver or applicable to other organs as well.

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

The A_{2A} adenosine receptor is involved in the regulation of addiction induced by different drugs of abuse. The specific role of A_{2A} receptors in the behavioural and neurochemical responses to morphine associated with its motivational properties were tested. The acute administration of morphine induced a similar enhancement of locomotor activity and antinociceptive responses in both A_{2A} knockout and control mice. However, the rewarding effects induced by morphine were completely blocked in A_{2A} KO mice. Also, naloxone did not induce place aversion in animals lacking A_{2A}. The results demonstrate that the rewarding and aversive effects associated with morphine abstinence were abolished in A_{2A} KO mice, supporting a differential role of the A_{2A} adenosine receptor in the somatic and motivational effects of morphine addiction. This provides evidence for the role of A_{2A} receptors as general modulators of the motivational properties of drugs of abuse (Castañé et al. 2008).

We have shown previously that mice lacking the adenosine A_{2A} receptor generated on a CD1 background self-administer more ethanol and exhibit hyposensitivity to acute ethanol. We have now shown that these A_{2A} KO mice display a reduced ethanol-induced conditioned place preference (CPP) and an increased sensitivity to the anxiolytic and locomotor stimulant effects of ethanol, but they did not show alteration in ethanol-induced conditioned taste aversion and locomotor sensitization. However, in A_{2A} KO mice on a C57BL/6J background, no difference was observed in terms of ethanol consumption and preference, ethanol-induced CPP and locomotor-stimulant effects. Nevertheless the A_{2A} agonist CGS 21680 reduced ethanol consumption and preference in C57BL/6J mice. Despite genetic background differences, the results show that inactivation of the A2a receptor leads to high ethanol consumption, increased sensitivity to the locomotor-stimulant/anxiolytic effects of ethanol and a decrease in ethanol-induced CPP (Houchi et al. 2008).

Long-term caffeine intake has been reported to decrease the susceptibility to convulsants in mice. We investigated the occurrence of seizures following long-term oral administration of caffeine in adenosine A_{2A} receptor knockout and control mice. Clonic seizures induced by acute pentylenetetrazol (PTZ) were significantly attenuated in KO mice and also reduced by a 14-day caffeine treatment in WT mice. We showed also a protecting effect of a 21-day caffeine treatment in WT mice against kindled seizures induced by PTZ in an increasing dose schedule. The protective effects against PTZ-induced seizures occurring when the adenosine A_{2A} receptor is absent or chronically blocked by caffeine is likely due to a decreased neuronal excitability in these conditions (El Yacoubi et al. 2008).

Peripheral nerve injury produces a persistent neuropathic pain state characterized by spontaneous pain, allodynia and hyperalgesia. The possible involvement of adenosine receptors in the development of neuropathic pain and the expression of microglia and astrocytes in the spinal cord after sciatic nerve injury was evaluated. Partial ligation of the sciatic nerve was performed in A_{2A} knockout mice and wild-type littermates. The development of mechanical and thermal allodynia, as well as thermal hyperalgesia was evaluated by using the von Frey filament model, the cold-plate test and the plantar test, respectively. In wild-type animals, sciatic nerve injury led to a neuropathic pain syndrome that was revealed in these three nociceptive behavioural tests. However, a significant decrease of the mechanical allodynia and a suppression of thermal hyperalgesia and allodynia were observed in $A_{2A}R$ deficient mice. Taken together, these results demonstrate the involvement of $A_{2A}Rs$ in the control of neuropathic pain (Bura et al. 2008).

Adenosine triphosphate has previously been shown to induce semi-mature human monocyte-derived dendritic cells (DC) through the $P2Y_{11}$ receptor. We showed that in mice, ATP and adenosine inhibited the production of IL-12p70 by bone marrow-derived DC (BMDC). In the absence of $P2Y_{11}$ receptor in mouse, the effects of adenine nucleotides on mouse DCs are mediated by their degradation product, adenosine, acting on the A_{2B} receptor (Ben Addi et al. 2008).

4. In vivo function of the CB_1 cannabinoid receptor

The endocannabinoid system is involved in the addictive processes induced by different drugs of abuse. We have tested the role of the CB_1 receptor in the pharmacological effects of 3,4-methylenedioxymethamphetamine (MDMA), a popular recreational drug. Acute MDMA administration increased locomotor activity, body temperature, and anxiogenic-like responses in wild-type mice, but these responses were lower or abolished in CB_1 knockout animals. MDMA produced similar conditioned place preference and increased dopamine extracellular levels in the nucleus accumbens in both genotypes. However, CB_1 knockout mice failed to self-administer MDMA at any of the doses used. These results indicate that CB_1 receptors play an important role in the acute prototypical effects of MDMA and are essential in the acquisition of an operant behavior to self-administer this drug (Touriño et al. 2008).

We have investigated further the involvement of the CB_1 receptor in the responses to stress. Stress is known to cause damage and atrophy of neurons in the hippocampus by deregulating the expression of neurotrophic factors that promote neuronal plasticity. The endocannabinoid system is involved in neuroprotection at both cellular and emotional levels. We showed that CB1 knockout mice exhibit an increased response to stress, including increased despair behavior and corticosterone levels in the tail suspension test, and decreased brain derived neurotrophic factor (BDNF) levels in the hippocampus. Local administration of BDNF in the hippocampus reversed the increased despair behavior of CB_1 knockout mice, confirming the role played by BDNF in the emotional impairment of these mice. No differences were found in the levels of other neurotrophic factors, NGF and NT-3, or the activity of the BDNF receptor and transcription factor CREB. These results suggest that the lack of CB_1 receptor results in an enhanced response to stress and deficiency in neuronal plasticity by decreasing BDNF levels in the hippocampus, leading to impairment in the responses to emotional disturbances (Aso et al. 2008).

The role of the CB_1 receptor in serotonin release in the hippocampus was investigated. Mouse hippocampal slices were preincubated with [^{3}H]serotonin and superfused with medium containing a serotonin reuptake inhibitor (citalopram hydrobromide). The cannabinoid receptor agonist WIN55,212-2 did not affect the resting [^{3}H]5-HT release, but decreased the evoked [^{3}H]5-HT efflux in wild-type mice. This effect was abolished by the selective CB_1 antagonists SR141716 and AM251. The inhibitory effect of WIN55,212-2 was also completely absent in hippocampal slices derived from CB_1 knockout mice. Selective degeneration of fine serotonergic axons by the neurotoxin parachloramphetamine reduced the uptake and evoked release of [^{3}H]5-HT, and eliminated the effect of WIN55,212-2. These data suggest that a subpopulation of non-synaptic serotonergic afferents express CB_1 receptors and activation of these CB_1 receptors leads to a decrease in 5-HT release (Balázsa et al. 2008).

The effect of WIN 55,212-2 were also investigated on excitatory postsynaptic currents (EPSCs) evoked by stimulation of Schaffer collaterals in CA1 pyramidal cells. WIN 55,212-2 reduced the amplitude of EPSCs in a dose-dependent manner. In rats and mice, this cannabinoid ligand inhibited excitatory synapses in two steps at the nM and muM concentrations. In CB_1 knockout animals, of under treatment with the CB_1 antagonist AM251, WIN 55,212-2 could still reduce the amplitude of EPSCs at μM but not nM concentrations. The inactive enantiomer, WIN 55,212-3, mimicked the effect of WIN 55,212-2 applied in high doses. The CB_1-independent effect of WIN 55,212-2 at glutamatergic synapses was abolished by the omega-conotoxin GVIA, but not with the omega-agatoxin IVA. These data suggest that, in the hippocampus, WIN 55,212-2 reduces glutamate release from Schaffer collaterals solely via CB_1 receptors in the nM concentration range, whereas in μM concentrations, WIN 55,212-2 suppresses excitatory transmission by an additional mechanism independent of CB_1, the blockade of N-type voltage-gated Ca^{2+} channels (Németh et al. 2008).

We showed that cannabinoid receptor agonists can be immunosuppressive and neuroprotective in models of multiple sclerosis. Immunosuppression was associated with a reduction of myelin-specific T cell responses, central nervous system infiltration and clinical signs. These effects were shown to be largely CB_1-dependent and occurred at doses inducing significant cannabimimetic effects. Lower, non-immunosuppressive doses of cannabinoids slowed the accumulation of nerve loss and disability, but failed to inhibit relapses. These results further highlights the neuroprotective potential of cannabinoids to slow the progression of multiple sclerosis.

5. 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. These include GPR37, GPR88, GPR85.

We have established cell lines coexpressing $G_{\alpha 16}$, apoaequorin and genes encoding the selected orphan receptors. To be able to monitor the expression at the cell surface of recombinant cells by FACS analysis, we have also fused some of the receptors (GPR88) at their N-terminus to an epitope tag. Finally, $G_{\alpha 16}$ is known to couple most receptors to the phospholipase C-calcium release pathway, but is not the optimal coupling protein for all receptors. The use of a chimeric G protein, made of the $G_{\alpha q}$ backbone, with the last 5 aminoacids originating from the $G_{\alpha i}$ sequence, can be a better partner to

couple to calcium mobilization some receptors naturally linked to the G_i pathway. Cell lines coexpressing apoaequorin, the $G_{\alpha qi5}$ chimera, and the receptors of interest were also established.

The cell lines expressing orphan receptors are used for the screening of biological activities in a library of fractions prepared from natural sources. Biological extracts are indeed expected to contain the natural ligands of orphan receptors, more particularly the naturally processed forms of peptides and proteins, containing necessary tertiary structures and post-translational modifications. Extracts, fractionated through a first step of HPLC, were made mainly from pig, rat and mouse tissues. Potential biological activities are purified using various multidimensional chromatographic procedures (reverse phase HPLC, ion exchange, heparin affinity) in order to obtain a purity level suitable for mass spectrometry and peptide sequencing (MALDI Q-TOF fragmentation analysis) and identify the primary structure of the peptide or protein.

Starting from a rat colon extract, a first step of fractionation on a strong anion exchange column in Tris/HCl buffer, using a NaCl concentration gradient, resulted in a potential activity peak for one of the orphan receptors tested. The positive fractions were pooled and used to perform a second dimension on a polycationic exchange column in ammonium acetate buffer. In this second step, we confirmed the presence of a specific activity for the receptor eluted between 150 and 230 mM NaCl. We are now optimizing a third dimension by testing different columns such as cationic exchange columns or reverse phase HPLC using C18 or C4 columns.

We have also obtained a knock out model for the GPR37 receptor, which is being bred on a C57Bl6 background, and are presently preparing a construct that will allow to inactivate the GPR88 gene in mouse ES cells.

6. Search for partners of olfactory receptors

The structure of olfactory receptors has been known for 15 years, but their functional expression is still a major problem, as mammalian ORs are poorly targeted to the cell surface in heterologous systems. Due to these limitations, only a few mammalian ORs have been characterized functionally to date. One of the most significant improvements reported over the last few years is the demonstration that the transmembrane proteins RTP1, RTP2 (both expressed specifically in olfactory neurons) and REEP1 contribute to the translocation of ORs to the plasma membrane, and promote their functional expression in mammalian cell lines (Saito et al. Cell 2004), allowing the design of more reliable functional assays. It is however our hypothesis that additional membrane or soluble proteins are required for the reconstitution of an efficient signalling complex in the knobs of olfactory neuron cilia, complexes that may resemble those found at neuronal synapses, in which receptors, transduction proteins and channels are organized by a number of chaperones and scaffolding proteins. We have started to explore further this hypothesis by using a proteomic approach. We have established a procedure for the homogenization of olfactory mucosa from mouse and the preparation of a fraction enriched in ciliary knobs. This enrichment has been demonstrated by the detection of the adenylate cyclase III protein, which is part of the signalling machinery proximal to the receptor. In order to purify further the protein complexes containing the receptors, it will be necessary to express a specific tagged olfactory receptor in all olfactory neurons. To this end, we have developed a transgenic mice that place the expression of a receptor labelled with a F5-tag under the control of tetracyclin responsive promoter, and bred these

mice with a strain expressing two other transgenes (OMP-TTA and G8-TTA), allowing the permanent expression of a given receptor in all olfactory neurons. Such expression is not achieved adequately using less sophisticated approaches, as a result of a complex control of receptor expression in olfactory neurons, involving apparently the coding sequence of the receptors themselves (Nguyen et al. Cell 131:1009-17, 2007). We now have several mice expressing the three transgenes and verified receptor expression in mice expressing OMP-TTA and TRE-F5-OR. As expected, we found that many but not all olfactory neurons do express the olfactory receptor labelled with an anti-Tag antibody. Co-labelling with anti-adenylate cyclase III antibody (ACIII) also indicated that these receptors were expressed in the cilia of olfactory neurons. We now plan to verify that triple transgenic mice indeed express the same olfactory receptor in all neurons and then prepare an OR-enriched fraction from these mice by immunoprecipitation with an anti-Tag monoclonal antibody. Once purified, we will screen by 2D-DIGE and 2D-LC the proteins over-represented in this fraction, as compared to a total membrane fraction of the same initial homogenate or as compared to samples prepared from wt mice. Proteins identified by mass spectrometry will be considered as part of signalling complexes and will be tested functionally in reconstituted systems in mammalian cell lines co-expressing ORs, Golf, and the cyclic-nucleotide-gated channel. We will also search for accessory proteins involved in cell surface targeting of OR by studying cellular localisation (using microscopy) of OR in cell lines co-expressing the newly identified accessory proteins and various olfactory receptors.

7. ChemR23

Chemerin is a potent chemotactic factor that was identified recently as the ligand of ChemR23, a G protein-coupled receptor expressed by mononuclear phagocytes, dendritic cells (DCs), and NK cells. Chemerin is synthesized as a secreted precursor, prochemerin, which is poorly active on ChemR23. However, prochemerin can be converted rapidly into a full ChemR23 agonist by proteolytic removal of a carboxy-terminal peptide. This maturation step is mediated by the neutrophil-derived serine proteases elastase and cathepsin G. We have now investigated proteolytic events that negatively control chemerin activity. We demonstrated that neutrophil-derived proteinase 3 (PR3) and mast cell (MC) chymase are involved in the generation of specific chemerin variants, which are inactive, as they do not induce calcium release or DC chemotaxis. Mass spectrometry analysis showed that PR3 specifically converts prochemerin into a chemerin form, lacking the last eight carboxy-terminal amino acids, and is inactive on ChemR23. Whereas PR3 had no effect on bioactive chemerin, MC chymase was shown to abolish chemerin activity by the removal of additional amino acids from its C-terminus. This effect was shown to be specific to bioactive chemerin (chemerin-157 and to a lesser extent, chemerin-156), as MC chymase does not use prochemerin as a substrate. These mechanisms, leading to the production of inactive variants of chemerin, starting from the precursor or the active variants, highlight the complex interplay of proteases regulating the bioactivity of this novel mediator during early innate immune responses (Guillabert et al. 2008).

We also contributed to the demonsration that chemerin-expressing plasmacytoid dendritic cells (pDCs) accumulate in white matter lesions and leptomeninges of the brain of multiple sclerosis patients, while chemerin was found in intralesional cerebrovascular endothelial cells. This suggests that the chemerin/ChemR23 system plays a role in the control of pDC recruitemnt in multiple sclerosis (Lande et al. 2008).

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.

CCR2/CCR5, CCR2/CXCR4 and CCR5/CXCR4 heterodimers. We reported previously the existence of negative binding cooperativity between the subunits of CCR2/CCR5 and CCR2/CXCR4 heterodimers (El-Asmar et al., 2004, Springael et al., 2006, Sohy et al., 2007). We extended these observations to heterodimers formed by CXCR4 and CCR5 demonstrating that specific agonists and antagonists of one receptor can compete allosterically for the binding of a specific tracer of the other when the two receptors are co-expressed. CCR2, CCR5 and CXCR4 form thus homodimers as well as heterodimers with one another, raising the question of their natural organization at the surface of immune cells expressing these three receptors endogenously. Using Bi-LC BRET assays, we demonstrated that hetero-oligomeric complexes containing simultaneously the three receptors are formed. Importantly, we also showed that specific antagonists of one receptor inhibit the binding of chemokines to the other receptor as a consequence of their heterodimerization, both in recombinant cell lines and primary leukocytes. This resulted in a significant functional cross-inhibition in terms of calcium mobilization and chemotaxis. Using the air pouch model in mice, we established that this trans-inhibition by antagonists has major consequences on the migration of cells in vivo. We showed that the CCR2 and CCR5 antagonist TAK-779 inhibits both lymphocytes and dendritic cells recruitment into the pouch in response to SDF-1α. This data support the new concept following which small-molecule antagonists can trans-modulate the function of receptors on which they do not bind directly, as the result of their heterodimerization, with important implications on the activities of chemokine receptor antagonists in vivo. We have started to study a larger range of antagonists in order to test whether trans-inhibition is a property shared by all antagonists of these receptors or restricted to some molecules only. From a general point of view, allosteric regulation across GPCR oligomeric interfaces is expected to greatly influence the practice of modern pharmacology. It will likely affect the design of drug discovery programs, which rely mostly on the overexpression of the receptor of interest in a cell line, thereby focusing on homo-oligomers and ignoring the potential effects of other partners.

Heterodimerization of ChemR23 with CXCR4 and CCR7. With the aim of further characterizing functional consequences of chemokine receptors dimerization, we also investigated the dimerization status of ChemR23. Like chemokine receptors, ChemR23 is expressed on leukocytes such as macrophages, dendritic cells as well as on a subset of NK cells. Using BRET, we showed that ChemR23 is able to form heterodimers with chemokine receptors CXCR4 and CCR7 constitutively and that this interaction results in a strong negative binding cooperativity. These results support the view that negative binding cooperativity takes also place across receptors that bind ligands structurally unrelated. We also showed on mouse BMDC expressing endogenously ChemR23 and CXCR4, that chemerin competed for SDF-1α binding and that this cross-inhibitory effect is specifically lost in cells generated from mice invalidated for ChemR23, thus demonstrating the functional relevance of ChemR23/CXCR4 dimerization in primary leukocytes.

Heterodimerization of CCR7 with CXCR4, CCR5 and CCR2. We also investigated the dimerization status of the chemokine receptor CCR7 and showed using BRET that CCR7 forms heterodimers with CCR2, CCR5 and CXCR4 constitutively. Similarly to what we reported for other chemokine heterodimers, we showed that the heterodimerization of CCR7 with CCR2 is associated with a “symmetrical” negative binding cooperativity, the ligand of each receptor being able to compete for the binding of radiolabelled tracer to the other. In contrast, CCR7 heterodimerization with CCR5 is linked to an “asymmetrical” negative binding cooperativity i.e. specific ligands of CCR7 being able to compete for radiolabelled

tracer to CCR5 while CCR5-specific ligands being unable to do so for tracer binding to CCR7. Finally, we showed that CCR7 heterodimerization with CXCR4 does not involve binding cooperativity, the ligands of each receptor composing the dimer being unable to compete for binding of radiolabelled tracer to the other. Among all the chemokine heterodimers we tested, this is the first case of receptors for which heterodimerization is not associated with negative binding cooperativity. In contrast, we showed that the functional response of CXCR4 and CCR5 is strongly reduced upon co-expression of CCR7. Expression of CCR7 had no major effect on the EC50 values of the dose-response curves but decreased drastically the maximal response. This decrease of response was however not linked to a reduced expression level of CCR5 or CXCR4 receptor as measured by FACS or saturation binding assay. The molecular mechanism underlying this phenomenon is not known for sure but might involve conformational change of CCR5 and CXCR4 receptors as the result of their interaction with CCR7. Interestingly, this negative effect of CCR7 was not detected in cells coexpressing CCR7 with CCR2 or ChemR23, suggesting that properties of receptors might vary greatly according to the partner with which CCR7 interacts.

We contributed also to studies showing synergistic effects of CC and CXC chemokines in chemotactic assays using monocytes (Gouwy et al. 2008). These synegistic effects likely involve post-receptor signaling cascades. The citrullination of the CXCL12, resulting in the inactivation of the chemokine was also reported (Struyf et al. 2008).

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Progress Report of the Research Group of

Dr. L. Ris

Université de Mons-Hainaut (UMH)

Dr. L. Ris

University of Mons-Hainaut
Laboratory of Neurosciences
Place du Parc, 20
B-7000 Mons
Tel.: +32 65 37 35 72
Fax: +32 65 37 35 73
laurence.ris@umh.ac.be

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.

Therefore, the long-lasting increase in synaptic strength, that can be triggered *in vitro* in hippocampal slices (thickness: 0.4 mm) artificially maintained alive, provides a biological model of memory at the elementary level. This type of synaptic plasticity is known as "Long-Term Potentiation" or LTP. When a long-lasting LTP (more than 4h) is triggered, its early phase (E-LTP) which lasts 1-2h, does not depend on protein synthesis. On the contrary, its late phase (L-LTP) strongly depends on a *de novo* protein synthesis. The mechanisms of E-LTP have been revealed; those of L-LTP are unknown, in particular because only a few of the proteins synthesized in response to the stimuli are not yet known.

Aim of the project

The primary goal of the project was to identify the proteins whose amount increases in the synaptic region and in the dendritic spines in particular during the late phase of L-LTP. The basic idea was to compare the relative quantities of several hundreds of proteins from the synaptoneurosomes originating from hippocampal slices where an L-LTP had been pharmacologically induced on one hand and from control hippocampal slices on the other hand.

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.

Problems solved during this first year

1. Isolation of the synaptoneurosomes

The first problem was to isolate the synaptoneurosomes from the very small samples we have to deal with (50 CA1 regions or more adequately 90 CA1 dendritic regions; Fig. 1A). Three protocols were tried: (1) ultracentrifugation using a sucrose gradient, (2) ultracentrifugation using a percoll gradient and (3) to push the sample through filters whose meshes were 5 µ wide. Probably due to the smallness of our sample, the first two procedures failed. The filter technique provided enough proteins.

We checked by electron microscopy that the isolated fraction did contain synaptoneurosomes (Fig. 1B).

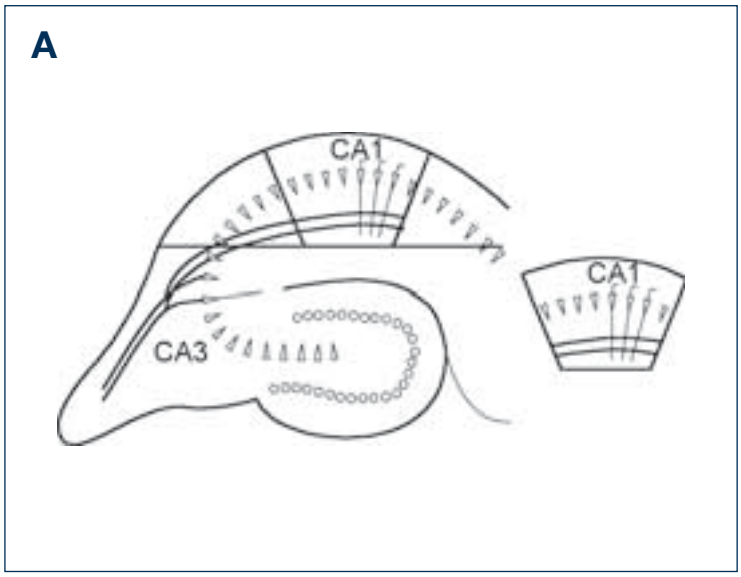

Fig. 1. A. Sketch of a hippocampal slice. The sampled area was either the CA1 region or the dendrites of this region.

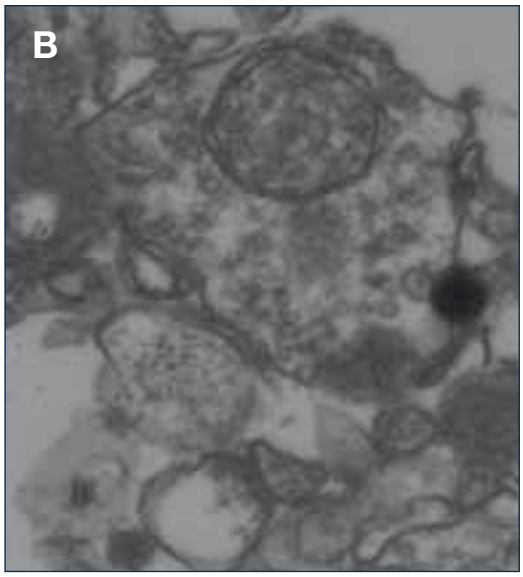

Fig. 1. B. Electron micrograph of synaptoneurosomes isolated from mouse hippocampus.

2. Checking that the synaptoneurosomal fraction does contain and is enriched in proteins known as synaptic

The proteins of the synaptosomal fraction were extracted using guanidium chloride 7M (Gu-HCl), a chaotropic agent. After removing of guanidium by dialysis, the extract was centrifugated. The proteins of the supernatant and of the pellet were separated by SDS-PAGE. We checked by mass spectrometry that among them were the NMDA receptor (NMDA R1), the AMPA receptor (GluR2 and 3), the mGluR2/3 receptor, CaMKIIα and PSD-95. We also checked by western blot that these proteins were enriched in the filtrate (in comparison with the homogenate) (Fig. 2).

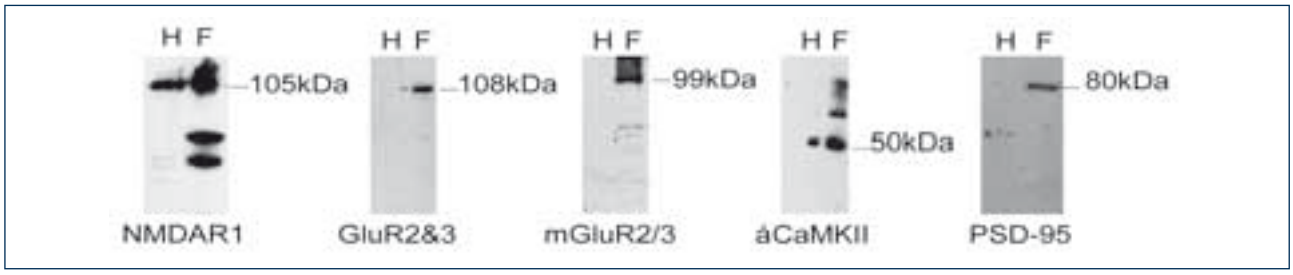

Fig. 2. Western profiles of homogenate (H) and filtrate (F).

3. Perfecting of the differential isotopic labeling

We perfected the technique of differential labeling of two large proteins samples from two cerebral cortex with succinimide labeled with C^{13} for one sample and with natural succinimide (C^{12}) for the other sample. The ratios of each couple of labeled peptides were in each case around 1, which proved that the method was valid in our hands.

4. First results

We then applied the technique to the preparation of interest: samples from the dendritic CA1 treated with forskolin – IBMX versus untreated samples. These two samples were labeled, mixed and then submitted to 2D-LC/MS-MS. Trypsin and endoglu digestion was done on the whole sample, without preliminary protein separation by SDS-PAGE. In this first trial, we found that L-LTP triggered chemically was accompanied by a significant up-regulation of 30 proteins (out of 272 identified ones) in the dendrites of the CA1 region. Not a single decrease was observed.

Progress Report of the Research Group of

Prof. dr. S.N. Schiffmann

Université Libre de Bruxelles (ULB)

Prof. dr. Serge N. Schiffmann

Laboratory of neurophysiology,
Department of neuroscience
Faculty of medicine
Free University of Brussels
808 route de Lennik - CP601
B-1070 Bruxelles
Tel.: +32 2 555 64 07 - +32 2 555 42 30
Fax: +32 2 555 41 21
sschiffm@ulb.ac.be

Senior collaborators:

Jean-Marie Vanderwinden
Alban de Kerckhove
David Gall

Direct and Indirect pathways in basal ganglia functions and disorders

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

Our aim was to generate transgenic mice allowing the study of the specific roles of striatopallidal or striatonigral neurons. We have obtained mice strains expressing the Cre recombinase under the control of the A_{2A} receptor promoter inserted in a BAC (bacterial artificial chromosome) to direct gene expression specifically in striatopallidal neurons (A2Ar-Cre). These lines 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) in order to determine whether they selectively expressed Cre in these striatopallidal neurons. Co-localisation experiments using anti-enkephalin antibody (Enk) and LacZ activity detection showed that >80% of the LacZ neurons are also Enk+. 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 D2 and A_{2A} receptors as demonstrated by single cell RT-PCR. Therefore, altogether, these results demonstrated that we have generated strains of transgenic mice with a specific Cre expression in the striatopallidal neurons (de Kerchove d'Exaerde et al., 2006; Durieux et al., 2008).

Thanks to the generation this specific A2Ar-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 diphteria toxin receptor (rosa26-lox-stop-lox-DTR mice). Through the stereotaxic injection of diphteria toxin, this results in the specific ablation of striatopallidal neurons in the full striatum or selectively in the accumbens nucleus (Durieux et al., 2008). This was the first genetic model allowing selective ablation of these striatopallidal cells. By using this strategy we showed that striatopallidal neuron loss in the entire striatum induces marked hyperlocomotion while surprisingly, restricted ablation in the nucleus accumbens increases amphetamine conditioned place preference. These results demonstrated the motor inhibitory role of striatopallidal neurons and an unexpected involvement in limiting the drug-reinforcement process. (Durieux et al., 2008)

The striatopallidal-GFP mice has been used as a tool to specifically isolate this population of striatal neurons by using dissociation and sorting by FACS and to specifically record them by using the patch clamp technique. We succeed in isolating green (striatopallidal) and red (striatonigral) neurons from individual mice with a very good rate of enrichment. In this frame, our aim is now to establish the gene expression profiles of these different populations by microarrays. RNA amplification and identification will be performed to validate the differential expression of some known genes (i.e. enkephalin , substance P, A_{2A} receptor). Despite the similar results published by a competing team (Lobo et al. 2006), we have identified a series of new genes which showed high differential expression in the striatopallidal neurons. This differential expression has been validated by using different techniques and their physiological relevance is currently studied using different knock down strategies. In parallel, we have extended our studies by examining the modifications of the specific gene expression profile in this striatopallidal population in pathological conditions.

We have crossed these "striatopallidal-Cre" mice with strains of "floxed" mice allowing the selective inactivation of genes in this population of neurons. The NMDA receptors seem to play a key role in reward and drug addiction (ventral striatum) and in motor learning (dorsal striatum). Moreover, NMDA receptors are supposed to be involved in synaptic plasticity at the cortico-striatal and cortico-accumbal synapses (LTP and LTD). Synaptic plasticity in these areas is strongly suggested to be the basis for motor learning and addiction. An important question is therefore the specific role of this receptor in

each subpopulation. The NR1 floxed ($NR1^{f/f}$) mice allowing a conditional inactivation of NR1 by the Cre recombinase have been double-crossed with our $A_{2A}R$ -Cre mice to obtain homozygous mice. The characterization of $A_{2A}R$-Cre/+ $NR1^{f/f}$ mice showed a selective decrease in NMDA receptor binding in the caudate-putamen and accumbens nucleus as compared to the cerebral cortex. Behavioural, electrophysiological and morphological characterizations of these mice are currently in progress.

Regulation of striatal neurons excitability and corticostriatal synaptic transmission

We previously studied the modulation of the glutamatergic corticostriatal transmission and plasticity by A_{2A} receptor (D'Alcantara et al., 2001). We now studied the mechanisms of the modulation exerted by D2 and A_{2A} receptors on the striatal neurons excitability and corticostriatal synaptic transmission in normal and hypodopaminergic conditions. By using the perforated patch configuration of the patch clamp technique in combination with peptide occlusion protocols, we showed that dopamine D2 receptor activation abolished the NMDA-induced down- to up-state transitions and hence striatal neurons excitability. Peptide occlusion showed that this effect occurs through a pathway involving a subtype of calcium channels (CaV1.3). This D2-mediated effect is fully reversed by co-stimulation of A_{2A} receptor although activation of the A_{2A} receptor is unable to modify the down- to up-state transitions (Azdad et al., 2008; Schiffmann et al., 2007; Ferré et al., 2008). Such effect was shown to be specific of the striatopallidal neuron population since it was only detected on GFP neurons in D2-GFP mice. This suggests that the action of A_{2A} receptor activation could be completely or partially due to an intramembrane interaction such as D2-A_{2A} heteromerization rather than to activation of an intracellular cascade. Experiments have been performed to test this hypothesis by using occlusion by specific competitive peptides blocking A_{2A}-D2 heteromerization. We showed that these peptides fully blocked the ability of A_{2A} receptor activation to counteract the D2 effect demonstrating the involvement of D2-A_{2A} heteromerization in this modulation and supporting for the first time in a physiological condition, the physiological relevance of this heteromerization (Azdad et al., 2008; Schiffmann et al., 2007; Ferré et al., 2008).

Stem cell graft in models of Huntington's disease

We have studied the migration and homing processes of neural stem cells (NSC) and mesenchymal stem cells (MSC) following their intracerebral grafts in vivo, in a rat model of Huntington's disease. We showed that both stem cells (NSC and MSC) exhibit a migration pattern significantly different in the degenerative condition as compared to intact brain (Bantubungi et al., 2008). Our results demonstrated that the host environment determine the final localisation of grated cells and hence their homing. We have identified a molecular mechanism involved in this homing process as the « Stem Cell Factor (SCF) – Kit receptor » system (Bantubungi et al., 2008). Indeed, we demonstrated that SCF expression is highly upregulated in the lesioned striatum as compared to the intact side and showed that NSC and MSC express the Kit receptor. Different technical approaches in vitro demonstrated that this SCF-Kit, ligand-receptor, system is functional in both cell types since recombinant SCF as well as protein extract from the lesioned striatum, increased phosphorylation of Kit and Erk, increased cell proliferation and cell migration. In vivo studies using either a blocking Kit antibody or Kit-deficient mice are currently running in order to demonstrate in situ the functional role of the SCF – Kit receptor system in these migration and homing processes.

We have also took part in the characterization of cortical neurons generated from embryonic stem cells by showing an intrinsic excitability and functional synaptic transmission in these cells (Gaspard et al., 2008)

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Progress Report of the Research Group of

Prof. dr. V. Timmerman

Universiteit Antwerpen (UA)

Principal Investigator:

Prof. Dr. Vincent Timmerman, PhD
Peripheral Neuropathy Group
VIB Department of Molecular Genetics
Institute Born Bunge
University of Antwerp

Co-investigator:

Prof. Dr. Peter De Jonghe, MD, PhD
Neurogenetics Group
VIB Department of Molecular Genetics
Institute Born Bunge
University of Antwerp
Division of Neurology
University Hospital Antwerpen

Molecular genetics and biology of Charcot-Marie-Tooth neuropathies

1. Research report:

Charcot-Marie-Tooth disease (CMT) is the most common neuromuscular disorder. It represents a group of clinically and genetically heterogeneous inherited neuropathies. We reviewed the results of molecular genetic investigations and the clinical and neurophysiological features of the different CMT subtypes (Barisic et al. 2008). The products of genes associated with CMT phenotypes are important for the neuronal structure maintenance, axonal transport, nerve signal transduction and functions related to the cellular integrity. Identifying the molecular basis of CMT and studying the relevant genes and their functions is important to understand the pathophysiological mechanisms of these neurodegenerative disorders, and the processes involved in the normal development and function of the peripheral nervous system. The results of molecular genetic investigations have impact on the appropriate diagnosis, genetic counseling and possible new therapeutic options for CMT patients.

Distal hereditary motor neuropathy (HMN) or the spinal form of CMT is a clinically and genetically heterogeneous group of disorders predominantly affecting the motor neurons in the peripheral nervous system. So far mutations in six different genes have been identified for autosomal dominant distal HMN; glycyl-tRNA synthetase (GARS), dynactin 1 (DCTN1), small heat shock 27 kDa protein 1 (HSPB1), small heat shock 22 kDa protein 8 (HSPB8), Berardinelli-Seip congenital lipodystrophy (BSCL2) and senataxin (SETX). In addition a mutation in the (VAMP)-associated protein B and C (VAPB) was found in several Brazilian families with complex and atypical forms of autosomal dominantly inherited motor neuron disease. We investigated the distribution of mutations in these seven genes in a cohort of 112 familial and isolated patients with a diagnosis of distal motor neuropathy and found nine different disease-causing mutations in HSPB8, HSPB1, BSCL2 and SETX in 17 patients of whom 10 have been previously reported. No mutations were found in GARS, DCTN1 and VAPB. The phenotypic features of patients with mutations in HSPB8, HSPB1, BSCL2 and SETX fit within the distal HMN classification, with only one exception; a C-terminal HSPB1-mutation was associated with upper motor neuron signs. Furthermore, we provided evidence for a genetic mosaicism in transmitting an HSPB1 mutation. This study, performed in a large cohort of familial and isolated distal HMN patients, clearly confirmed the genetic and phenotypic heterogeneity of distal HMN and provided a basis for the development of algorithms for diagnostic mutation screening in this group of disorders (Dierick and Baets et al. 2008).

We also described a family with an unusual congenital lower motor neuron disorder with significant but static muscle weakness predominantly affecting the lower limbs. The proband had *talipes equinovarus* and congenital hip contractures and did not walk until 19 months of age. Lower-extremity predominant, primarily proximal weakness was identified on assessment at three years. Over a 20 year follow-up there has been no clinical progression. The proband has a four-year-old daughter with very similar clinical findings. Electromyography and muscle biopsy suggest reduced numbers of giant normal duration motor units with little evidence of denervation or reinnervation. Dominant congenital spinal muscular atrophy (SMA) predominantly affecting the lower limbs is rarely described. We proposed that the disorder is due to a congenital deficiency of motor neurons (Reddel et al. 2008)

Autosomal recessive Charcot-Marie-Tooth syndrome (AR-CMT) is often characterised by an infantile disease onset and a severe phenotype. Mutations in the ganglioside-induced differentiation-associated protein 1 (GDAP1) gene are thought to be a common cause of AR-CMT. Mutations in the periaxin (PRX) gene are rare. They are associated with severe demyelination of the peripheral nerves and sometimes lead to prominent sensory disturbances. To evaluate the frequency of GDAP1 and PRX mutations in early onset CMT, we examined seven AR-CMT families and 12 sporadic CMT patients, all presenting with progressive distal muscle weakness and wasting. In one family also prominent sensory abnormalities and sensory ataxia were apparent from early childhood. In three families we detected four GDAP1 mutations (L58LfsX4, R191X, L239F and P153L), one of which is novel and is predicted to cause a loss of protein function. In one additional family with prominent sensory abnormalities a novel homozygous PRX mutation was found (A700PfsX17). No mutations were identified in 12 sporadic cases. Our study suggested that mutations in the GDAP1 gene are a common cause of early-onset AR-CMT. In patients with early-onset demyelinating AR-CMT and severe sensory loss PRX is one of the genes to be tested (Auer-Grumbach et al. 2008).

Autosomal recessive demyelinating CMT4H manifests with early onset, severe functional impairment, deforming scoliosis and myelin outfoldings in the nerve biopsy. Mutations in the *FGD4* gene encoding the Rho-GTPase guanine-nucleotide-exchange-factor frabin were reported in 5 families. We characterized a novel mutation in *FGD4* and described the related phenotype in a 20 year-old female born from healthy consanguineous parents and affected with early-onset peroneal muscular atrophy syndrome. Mutational analysis of *FGD4* was performed by direct sequencing of genomic DNA. Transcriptional analysis was done by reverse transcriptase-PCR on leukocyte RNA. The proband disclosed a moderately severe, scarcely progressive CMT, markedly slowed nerve conduction velocities, and a demyelinating neuropathy characterized by prominent myelin outfoldings. Mutational analysis disclosed a c.1762-2a>g transition in the splice-acceptor site of intron 14 which was predicted to cause a truncated frabin (p.Tyr587fsX14). This finding confirms the genetic heterogeneity of *FGD4*, demonstrates that CMT4H has as variable functional impairment, and suggests that frabin plays a crucial role during myelin formation (Fabrizi, et al. in press).

The purpose of another study was to prospectively assess magnetic resonance imaging (MRI) findings of lower limb musculature in an axonal CMT disease (CMT2) pedigree due to mutation in the dynamin 2 gene (DNM2). The series comprises a proband patient aged 55 years and her two affected daughters aged 32 and 23. MRI included T1- and fat suppressed T2-weighted spin-echo sequences. MRI also showed extensive fatty infiltration of all calf muscle compartments with relative preservation of the deep posterior one. Fatty muscle infiltration increased distally in 19 out of 66 (23%) visualized calf muscles in the three patients, but this percentage increased to 64% in the youngest and least severe patient. Muscle edema without contrast enhancement was present in 23% of calf muscles. There was massive fatty atrophy of foot musculature. We concluded that MRI studies accurately depict lower limb muscle involvement in CMT2 caused by DNM2 mutation (Gallardo et al. 2008).

Congenital insensitivity to pain with anhidrosis (CIPA) is an autosomal recessive disorder caused by mutations in the neurotrophic tyrosine receptor kinase 1 (NTRK1) gene, which encodes the receptor for nerve growth factor. We reported the clinical course of a 7-year-old girl with CIPA and proven NTRK1 mutation. 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 patient was treated with regular administration of intravenous immunoglobulins. Conservative treatment of the recurrent left hip dislocation by cast

immobilization and bracing was implemented to stabilize the joint. The implication of the immune system of the reported patient broadened the clinical phenotype associated with NTRK1 mutations (Kilic et al. 2008).

Finally, we characterized a patient with 46XY gonadal dysgenesis and peripheral neuropathy. Examination of a sural nerve biopsy specimen revealed an axonal neuropathy with pronounced axonal loss, limited signs of axonal regeneration and no minifascicle-formation. A normal male karyotype was found (46XY) without micro-deletions in the Y chromosome. No mutations were found in the Sex-Determining-Region-Y (SRY) gene, Peripheral Myelin Protein 22 (PMP22), Myelin Protein Zero (MPZ), Gap-Junction protein Beta 1 (GJB1), Mitofusin 2 (MFN2) or Desert Hedgehog Homolog (DHH). The absence of minifascicle formation and of a mutation in DHH in this patient with gonadal dysgenesis and peripheral neuropathy expand the clinical and genetic heterogeneity of this rare entity (Baets et al. in press).

2. Research Activities:

Articles in International Journals with acknowledgement to GSKE

- Dierick,I.*, Baets,J.*, Irobi,J., Jacobs,A., De Vriendt,E., Deconinck,T, Merlini,L., Van den Bergh,P., Milic-Rasic,V., Robberecht,W., Fischer,D.F., Juntas Morales,R., Mitrovic,Z., Seeman,P., Mazanec,R., Kochañski,A., Jordanova,A., Auer-Grumbach,M., Helderman-van den Enden,A.T.J.M., Wokke,J.J.H., Nelis,E., De Jonghe,P., Timmerman,V.: Relative contribution of mutations in genes for autosomal dominant distal hereditary motor neuropathies: a genotype - phenotype correlation study. Brain 131(Pt 5): 1217-1227 (2008) (PMID: 18325928) (I.F.: 8.568): * equal contribution for the first authors.
- Gallardo,E., Claeys,K.G., Nelis,E., García,A., Canga,A., Combarros,O., Timmerman,V., De Jonghe,P., Berciano,J.: Magnetic resonance imaging findings of leg musculature in Charcot-Marie-Tooth disease type 2 due to dynamin 2 mutation. Journal of Neurology 255(7): 986-992 (2008) (PMID: 18560793) (I.F.: 2.477)
- Barisic,N., Claeys,K., Sirotkovic-Skerlev,M., Löfgren,A., Nelis,E., De Jonghe,P., Timmerman,V.: Charcot-Marie-Tooth Disease: A Clinico-genetic Confrontation. Annals of Human Genetics 72: 416-441 (2008) (PMID: 18215208) (I.F.: 2.307)
- Auer-Grumbach,M., Fischer,C., Papic,L., John,E., Plecko,B., Bittner,R.E., Bernert,G., Pieber,T., Miltenberger,G., Schwartz,R., Windpassinger,C., Grill,F., Timmerman,V., Speicher,M., Janecke,A.R.: Two Novel Mutations in the GDAP1 and PRX Genes in Early Onset Charcot-Marie-Tooth Syndrome. Neuropediatrics 39(1): 33-38 (2008) (PMID: 18504680) (I.F.: 1.225)
- Sebnem,S., Ozturk,R., Sarisozen,B., Rotthier,A., Baets,J., Timmerman,V.: Humoral immunodeficiency in congenital insensitivity to pain with anhidrosis. Neurogenetics 2008 (PMID: 19089473)(Epub ahead) (I.F.: 4.281)

Other topic related articles in international journals

- Young,P., De Jonghe,P., Stögbauer,F., Butterfass-Bahloul,T.: Treatment for Charcot-Marie-Tooth disease. Cochrane Database of Systematic Reviews 1: CD006052 (2008) (PMID: 18254090) (I.F.: 4.654)
- Beetz,C., Schüle,R., Deconinck,T, Tran-Viet,K-N, Zhu,H., Kremer,B.P.H., Frints,S.G.M., van Zelst-Stams,W.A.G., Byrne,P., Otto,S., Nygren,A.O.H., Baets,J., Smets,K., Ceulemans,B., Dan,B., Nagan,N., Kassubek,J., Klimpe,S., Klopstock,T., Stolze,H., Smeets,H.J.M., Schrander-Stumpel,C.T.R.M., Hutchinson,M., van de Warrenburg,B.P., Braastad,C., Deufel,T., Pericak-Vance,M., Schöls,L., De Jonghe,P., Züchner,S.: REEP1 mutation spectrum and genotype/phenotype correlation in hereditary spastic paraplegia type 31. Brain 31(Pt 4): 1078-1086 (2008) (PMID: 18321925) (I.F.: 8.568)
- Reddel,S., Ouvrier,R., Nicholson,G., Dierick,I., Irobi,J., Timmerman,V., Ryan,M.M.: Dominant non-progressive congenital spinal muscular atrophy: a primary developmental deficiency of motor neurons Neuromuscular Disorders 18(7): 530-535 (2008) (PMID: 18579380) (I.F.: 2.667)
- Haberlova,J., Claeys,K., Zámecník,J., De Jonghe,P., Seeman,P.: Extending the clinical spectrum of SPG3A mutations to a very severe and very early complicated phenotype. Journal of Neurology 255(6): 927-928 (2008) (PMID: 18446315) (I.F.: 2.477)
- De Weerdt,A., Claeys,K., De Jonghe,P., Ysebaert,D., Chapelle,T., Roeyen,G., Jorens,P.G.: Tacrolimus-related polyneuropathy: Case report and review of the literature. Clinical Neurology and Neurosurgery 110(3): 291-294 (2008) (PMID: 18055100) (I.F.: 1.553)

Articles in National Journals

- Irobi,J.: A molecular genetic update of inherited distal motor neuropathies. Verhandelingen - Koninklijk academie voor geneeskunde van België 70(1): 25-46 (2008)

Articles in International Journals, in press

- 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, in press.
- Hornemann,T., Penno,A., Richard,S., Nicholson,G., van Dijk,F.S., Rotthier,A., Timmerman,V., von Eckardstein,A.: Decision on a systematic comparison of all mutations in Hereditary Sensory Neuropathy Type I (HSAN I) reveals that the G387A mutation is not disease associated. Neurogenetics, in press.
- 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, in press.

Articles in Books, in press

- Baets, J., Hanemann, C., De Jonghe, P.: Inherited Peripheral Neuropathies. In: Encyclopedia of Molecular Mechanisms of Disease, Lang, Florian (Ed.), ISBN: 978-3-540-67136-7 (http://www.springer.com/biomed/molecular/book/978-3-540-67136-7).

Scientific Prizes:

- None

Awards and fellowships:

- Ricardo Gonçalves: Anacor Travel Fellowship to attend the AARS 2008 conference, Les Pensières, Lac Annecy, France; September 7-11, 2008
- Sophie Janssens: FWO travel fellowship to attend the "9th International Congress for Neuroimmunology" Forth Worth, Texas, USA, October 26-30, 2008
- Joy Irobi: fellowship to attend an Advanced Course Symposium and Practical Training on "Lentiviral vectors: concepts, practice, hope and reality", University Evry Val d'Essonne, Evry, France, June 29-July 5, 2008

PhD theses:

- None

Master theses:

- Kristof Van Avondt: "The role of the innate immune response in acute peripheral neuropathies", Masterthesis in Biochemistry (University of Antwerp), 2007-2008, supervisor: Sophie Janssens.

Bacherlor theses:

- Kristien Peeters: Fijnmappen van de locus voor de ziekte van Charcot-Marie-Tooth (CMT2G). Scriptie voorgelegd tot het behalen van de graad van Bachelor in de Biochemie en Biotechnologie. Promotor: Vincent Timmerman, Begeleiders: Annelies Rotthier.
- Yannick Waumans: Moleculaire karakterisatie van dominant intermediaire vorm van de ziekte van Charcot-Marie-Tooth type D, op zoek naar een gemeenschappelijke voorouder. Scriptie voorgelegd tot het behalen van de graad van Bachelor in de Biochemie en Biotechnologie. Promotor: Albena Jordanova, Begeleiders: Els De Vriendt en Jonathan Baets.

Invited Lectures at international meetings:

- Vincent Timmerman: "Are ubiquitously expressed genes good candidates for CMT neuropathies?", Inaugural International Neuromuscular Conference, UCL Institute of Child Health, London, 1-2 February 2008.
- Vincent Timmerman: "CMT genes and proteins: what we are learning for understanding peripheral neuropathies?" CMT workshop at the 18th Meeting of the European Neurological Society, Nice, 7-11 June 2008
- Vincent Timmerman: "Genetic and cell heterogeneity of Charcot-Marie-Tooth disease", International Symposium on Rare Diseases, Valencia, November 16-18, 2008

Invited Lectures at national meetings:

- Vincent Timmerman: Overzicht van het CMT onderzoek, Nationale Studie- en Contactdag van CMT België v.z.w, Antwerpen, 8 november 2008

Slide presentations selected at international meetings:

- Berciano, J., Gallardo, E., Claeys, K., García, A., Canga, A., Combarros, O., Timmerman, V., De Jonghe, P: Phenotypic characterisation of Charcot-Marire-Tooth disease type 2 associated to a novel dynamin 2 mutation. Journal of Neurology, 2008;255:suppl. 2:O74, 18th Meeting of the European Neurological Society, Nice, 7-11 June 2008
- Holmgren, A., De Winter, V., Dierick, I., Timmerman, V., Irobi, J.: Mutant HSPB8 and HSPB1 impairs formation of stable neurofilament network, International Symposium on Rare Diseases, Valencia, November 16-18, 2008
- Gonçalves, R., Storkebaum, E., Godenschwege, T., Nangle, L., Jacobs, A., Bosmans, I., Ooms, T., Yang, X.-L., Schimmel, P., Norga, K., Timmerman, V., Callaerts, P., Jordanova, A.: From YARS mutations to a peripheral neuropathy, a flying perspective. International Conference on Aminoacyl-tRNA synthetases: From Basic Mechanisms to System Biology; Les Pensieres, Lac Annecy France; September 7-11, 2008

Slide presentations selected at national meetings:

– none

Posters presentations at international meetings:

– Irobi, J., Krishnan, J., Almeida-Souza, L., Dierick, I., Ceuterick-de Groote, C., Van Den Bosch, L., Timmermans, J.P., Robberecht, W., De Jonghe, P., Janssens, S., Timmerman, V. Mutant heat shock protein HSPB8 induces aggregation and a pro-apoptotic phenotype in distal motor neuropathy. The 58th Annual Meeting of The American Society of Human Genetics in Philadelphia, Pennsylvania, to be held from Tuesday, November 11, through Saturday, November 15, 2008, at the Pennsylvania Convention Center.
– Irobi, J., Krishnan, J., Almeida-Souza, L., Dierick, I., Ceuterick-de Groote, C., Van Den Bosch, L., Timmermans, J.P., Robberecht, W., De Jonghe, P., Janssens, S., Timmerman, V. Mutant heat shock protein HSPB8 induces aggregation and a pro-apoptotic phenotype in distal motor neuropathy. Society for Neuroscience, SFN annual meeting, Nov. 15–19, 2008, Washington.
– Storkebaum, E., Gonçalves, R., Godenschwege, T., Nangle, L., Jacobs, A., Bosmans, I., Ooms, T., Yang, X.-L., Schimmel, P., Norga, K., Timmerman, V., Callaerts, P., Jordanova, A.:: DI-CMTC fly – the first Drosophilla model for inherited peripheral neuropathy. Society for Neuroscience, Washington, 2008.
– Van Avondt, K., Goethals, S., Jacobs, A., Timmerman, V., Janssens, S.: Induction of TLR expression in the peripheral nerve. Journal of Neuroimmunology 203(2): 191 (2008) 9th International Congress for Neuroimmunology, Forth Worth, Texas, USA, Oct 26-30, 2008.
– Almeida-Souza, L., Goethals, S., Heyrman, L., Goossens, D., Del-Favero, J., Timmerman, V., Janssens, S.: Generation of a human neuronal Flp-in host cell line. Molecular and Cellular Mechanisms of Axon Degeneration, Woods Hole, MA, USA, October 26th-28th 2008.
– Rotthier, A., Baets, J., Jacobs, A., De Vriendt, E., Jordanova, A., De Jonghe, P., and Timmerman, V.: Mutation analysis of genes for hereditary sensory and autonomic neuropathies: Identification of new mutations and a genotype-phenotype correlation study; Inherited Neuromuscular Diseases: Translation from Pathomechanisms to Therapies; International Symposium on Rare Disease, Valencia Spain, November 16th - 18th 2008

Posters presentations at national meetings:

– Dierick, I., Baets, J., Irobi, J., De Jonghe, P., Timmerman V.: Mutation analysis of HSPB1, HSPB8, BSCL2, GARS, DCTN1, SETX and VAPB in a large cohort of patients with distdal (hereditary) motor neuropathy; poster presentation, VIB seminar 2008, Blankenberge, March 6, 2008.
– Souza, L.C.A., Janssens, S., Timmerman, V: Searching for the pathomechanism of peripheral neuropathies related genes through a differential interaction-based technology: A methodology proposal. poster presentation, VIB seminar 2008, Blankenberge, March 6, 2008.
– Dierick, I., Baets, J., Irobi, J., Jordanova, A., De Jonghe, P., Timmerman, V.: Relative contribution of mutations in genes for autosomal dominant distal hereditary motor neuropathies: a genotype - phenotype correlation study. poster presentation, 8th annual meeting Belgian Society of Human Genetics, April 5th 2008
– Irobi, J., Krishnan, J., Almeida-Souza, L., Dierick, I., Ceuterick-de Groote, C., Van Den Bosch, L., Timmermans, J.P., Robberecht, W., De Jonghe, P., Janssens, S., Timmerman, V.: Mutant heat shock protein HSPB8 induces aggregation and a pro-apoptotic phenotype in distal motor neuropathy. Lentiviral vectors: concepts, practice, hope and reality, Advanced Course Symposium and Practical Training. University Evry Val d'Essonne, Evry, France, June 29-July 5, 2008.
– Irobi, J., Krishnan, J., Almeida-Souza, L., Dierick, I., Ceuterick-de Groote, C., Van Den Bosch, L., Timmermans, J.P., Robberecht, W., De Jonghe, P., Janssens, S., Timmerman, V.: Distal hereditary motor neuropathy caused by mutant HSPB8 reduced cell viability and induced protein aggregation VIB Seminar 2008. Blankenberge, March 6-7, 2008,

Patents:

– None

Progress Report of the Research Group of

Prof. dr. C. Van Broeckhoven

Universiteit Antwerpen (UA)

Principal Investigator

Prof. Dr. Christine Van Broeckhoven, PhD DSc
VIB – Department of Molecular Genetics
University of Antwerp – CDE
Universiteitsplein 1
2610 Antwerpen
www.molgen.ua.ac.be
E-mail: christine.vanbroeckhoven@ua.ac.be

Co-Investigators

Prof. Dr. Marc Cruts, PhD
E-mail: marc.cruts@ua.ac.be

Prof. Dr. Samir Kumar-Singh, MD PhD
E-mail: samir.kumarsingh@molgen.vib-ua.be

Table of contents

Progranulin in Neurodegenerative Dementia: Genetic, Functional and Neuropathological Characterization

The 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

1. PGRN null-mutations in FTLD and related neurodegenerative diseases

Worldwide, 63 heterozygous mutations were identified in 163 families, all leading to loss of functional GRN, implicating a haploinsufficiency mechanism (Gijselinck et al., 2008a). Since whole gene deletions also lead to the loss of a functional allele, we performed systematic quantitative analyses of *PGRN* in a series of 103 Belgian FTLD patients. We identified in one patient (1%) a genomic deletion that was absent in 267 control individuals. The deleted segment was between 54 and 69 kb in length and comprised *PGRN* and two centromeric neighboring genes *RPIP8* and *SLC25A39*. The patient presented clinically with typical FTD without additional symptoms, consistent with haploinsufficiency of *PGRN* being the only gene contributing to the disease phenotype. This study demonstrates that reduced *PGRN* in absence of mutant protein is sufficient to cause neurodegeneration and that previously reported *PGRN* mutation frequencies are underestimated. Together with the previously described null mutations in 11 patients, *PGRN* mutations account for 11.7% of all FTLD patients (Gijselinck et al., 2008b).

Mutations in *PGRN* might influence susceptibility to a wider range of neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) (Cruts and Van Broeckhoven, 2008). AD and FTLD are two frequent forms of primary neurodegenerative dementias. Despite distinctive clinical diagnostic criteria for both brain disorders, differential diagnosis is often complicated by

overlapping symptomatology. As we learn more about brain pathology and genetic makeup underlying these dementia disorders, evidence is accumulating for a clinical, pathologic, and genetic spectrum of neurodegenerative brain diseases in which AD and FTLD occur along one continuum. This has important implications for molecular diagnostic testing and genetic counseling of patients with dementia (van der Zee et al., 2008). Therefore, we assessed whether *PGRN* also contributes to genetic risk for AD in an extended Belgian AD patient group (n = 779, onset age 74.7 ± 8.7 years). We performed a mutation analysis of the *PGRN* coding region and assessed the effect of missense mutations using *in silico* predictions and protein modeling. We observed seven missense mutations in eight patients (1.3%). Convincing pathogenic evidence was obtained for two missense mutations, Cys139Arg and Pro451Leu, affecting PGRN protein folding and leading to loss of PGRN by degradation of the misfolded protein. In addition, using logistic regression analysis and gene-based haplotype association analysis, we showed that *PGRN* haplotypes were associated with increased risk for AD. Our data support a role for *PGRN* in patients with clinically diagnosed AD (Brouwers et al., 2008). Further, we hypothesize that at least some *PGRN* missense mutations might lead to loss of functional protein. Whether the underlying pathology in our cases proves to be AD, FTLD, or a combination of both must await further investigations.

Because of its apparent role in neuronal survival, we argued that *PGRN* might also contribute to PD pathogenesis. We screened *PGRN* exons for mutations in 255 patients with PD and 459 control individuals by direct genomic sequencing. In patients we identified four missense mutations of which Asp33Glu and Arg514Met were absent in control individuals. At this stage and in the absence of functional data, it remains unclear whether Asp33Glu and Arg514Met are biologically relevant to PD pathogenesis in the mutation carriers. Genetic association of *PGRN* with risk for PD was assessed using single nucleotide polymorphisms (SNPs) across the gene. Single SNP and haplotype analyses did not detect significant associations with PD. Our results therefore do not support a major role for *PGRN* in the genetic etiology of PD (Nuytemans et al., 2008).

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 relatives (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 diagnosing and early detection of FTLD-U caused by *PGRN* null mutations, and provided the first *in vivo* evidence that at least some missense mutations in *PGRN* may lead to a (partial) loss of *PGRN* (Sleegers et al., in press).

2. Genes modifying the variable onset age of FTLD associated with PGRN null mutations

The high variability in onset age and age-dependent penetrance suggests that the *PGRN* pathway is highly susceptible to modulating factors that might be exploited to delay the disease processes (Cruts and Van Broeckhoven, 2008). We performed a genome-wide linkage scan for genes modifying the variable onset age in the extended Belgian founder family DR8, segregating the *PGRN* 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 (Sleegers et al., 2009). 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.

3. Construction of PGRN constitutive and conditional knock-out and PGRN overexpression mice

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. Genotyping performed on PCR as well as Southern blotting on genomic DNA showed a successful knockout. In addition, neo-containing floxed mice are also being bred with flippase (FLP)-expressing mice (ACTFLPe deletor; Jackson Laboratories) to generate constitutive Neo-deleted floxed mice. These mice will be further crossbred with forebrain Cre-expressing lines (CamKIIa-cre) as well as with GFAP-cre and Thy-1-cre lines (all from Jackson Laboratories) to study the effect of region- or cell-specific conditional *Grn* ablation. So far, we have successfully bred $Grn^{-/-}$ mice up to 6 months of age. Experiments towards a full characterization of these mice are currently underway (Wils *et al.*, in preparation).

We also generated mice overexpressing human wild type *PGRN*. Full-length coding part of *PGRN* derived from lymphoblasts from healthy individuals was cloned in pDONR221 vector (Gateway, Invitrogen). An *ApaI/HindIII* restricted fragment containing the open reading frame of the PGRN gene was subcloned under the control of a modified murine Thy-1 promoter (mTUB; JSW Research). The construct was linearized and injected into male pronuclei of Bl6/SJL mice. Several transgenic pups were born. Transgenic expression in brain was studied by semi-quantitiative, qRT-PCR analysis and protein immunoblotting. Based on these data, two transgenic mice lines with > 4 times expression of transgenic PGRN compared to non-transgenic littermate controls were selected and being analyzed together with $Grn^{-/-}$ mice (Wils *et al.*, in preparation).

4. Elucidate the role of PGRN in mediating cell survival in *in vitro* models

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. We first showed that PGRN is predominantly localized in the cell secretory compartment and pulse chase experiments showed that within 6 hours, > 90% of freshly formed PGRN is secreted in cell supernatants as post-translationally modified, higher-MW PGRN. A small fraction, however, is retained within cells and is localized in fine cellular extensions. Together, these data suggest a role of PGRN in cell-signaling.

TDP-43 on the other hand localizes predominantly in the nucleus and even in the absence of a NLS-signal, TDP-43 and PGRN do not co-localize suggesting that there is no direct major interaction between these two proteins. Secondly, it was recently shown that siRNA-mediated down regulation of PGRN leads to caspase-3 activation and increased TDP-43 cleavage in neuroglial H4 cells. In our studies, while stable overexpression of PGRN increased cell proliferation, PGRN down regulation did not lead to reduced cell survival or induction of apoptosis in diverse cell lines such as HEK293 cells, N2a cells, H4 neuroglioma cells, and mouse embryonic fibroblasts derived from Grn knockout mice, suggesting that PGRN is well compensated in non-neuronal cells. Nevertheless, 2D-PAGE analysis also showed that TDP-43 is phosphorylated at many distinct sites and the caspase-mediated TDP-43 cleavage lead to several distinct fragments, including some highly basic and potentially aggregatable ones. We also studied physiological and pathological TDP-43 processing and showed that TDP-43 is constitutively processed in multiple human cell lines by non-caspase endoproteases with accumulation of ≈20–30 kDa N-terminal TDP-43 derivatives. Caspase activation reduces these ≈20–30 kDa N-terminal derivatives, and predominantly cleaves TDP-43 after residue 89 to produce ≈35 kDa C-terminal and ≈10 kDa N-terminal fragments; however, these do not represent major, pathologically-deposited TDP-43 derivatives (Kleinberger et al., under review).

Personnel

Principal Investigator

Prof. Dr. Christine Van Broeckhoven, PhD, DSc

Co-Investigators

Prof. Dr. Marc Cruts, PhD
Prof. Dr. Samir Kumar-Singh, MD PhD

Personnel paid by GSKE

PhD student
Hans Wils (01/10/2008)

Personnel involved in the project

- Staff Scientists
  Kristel Sleegers, Jessie Theuns
- Scientists
  Ahmed Amina (01/12/2008), Brouwers Nathalie, Gijselinck Ilse, Kleinberger Gernot, Nuytemans Karen, Pirici Nicolae Daniel, Sleegers Kristel, van der Zee Julie, Van Langenhove Tim, Wils Hans
- Technologists
  Cuijt Ivy, Joris Geert, Maes Githa, Corsmit Ellen
- Research nurses
  Mattheijssens Maria, Peeters Karin
- Diagnostics
  Marleen Van den Broeck

Publications

- Brouwers,N., Sleegers,K., Engelborghs,S., Maurer-Stroh,S., Gijselinck,I., van der Zee,J., Pickut,B.A., Van den Broeck,M., Mattheijssens,M., Peeters,K., Schymkowitz,J., Rousseau,F., Martin,J.J., Cruts,M., De Deyn,P.P., and Van Broeckhoven,C. (2008). Genetic variability in progranulin contributes to risk for clinically diagnosed Alzheimer disease. Neurology *71*, 656-664.
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Scientific activities

PhD theses

Name	Supervisor / Co-Supervisor	Title
Ilse Gijselinck 08/09/2008	C. Van Broeckhoven M. Cruts	'Molecular genomics of tau-negative, ubiquitin-positive frontotemporal lobar degeneration'
Nathalie Brouwers 12/12/2008	C. Van Broeckhoven K. Sleegers	'Molecular genetic analysis of Alzheimer disease'

MSc theses

Name	Supervisor / Co-supervisor	Title
Caroline Van Cauwenberghe	S. Kumar-Singh G. Kleinberger	Introduction to immunocytochemistry and western blotting techniques in FTLD
Tim Van Langenhove	C. Van Broeckhoven J. van der Zee	Genetic analysis for the valosin containing protein gene in Belgian frontotemporal lobar degeneration patients and clinico-pathological characterization of mutation carriers

Professional bSc theses

Name	Supervisor / Co-supervisor	Title
Jolien Brys	C. Van Broeckhoven J. van der Zee	Mutation screening of the microtubule associated protein tau (MAPT) and progranulin gene (PGRN) in Alzheimer and frontotemporal dementia patients.

Honors and prizes

- **C. Van Broeckhoven**: Distinguished Lecturer in Neuroscience and Aging at the National Institute on Aging IRP in Baltimore, USA, September 16, 2008

Awards

- **J. van der Zee:** Travel Fellowship of the Travel Fellowship Committee to attend the 11th International Conference on Alzheimer's disease (ICAD), Chicago, Illinois, USA, July 26-31, 2008
- **J. van der Zee**: Travel Fellowship to attend the 6th International Conference on FTD, Rotterdam, The Netherlands, September 3-5, 2008
- **K. Sleegers**: Travel Fellowship to attend the 6th International Conference on FTD, Rotterdam, The Netherlands, September 3-5, 2008

Chair and organizational activities

- **C. Van Broeckhoven:** 12th International Congress of Parkinson's disease and Movement Disorders: **Chair**: Session: Frontotemporal lobar degeneration, Chicago, USA, June 22-26 (2008)
- **C. Van Broeckhoven:** 6th International Conference on Frontotemporal Dementias, **International Advisory Board,** Rotterdam, the Netherlands, September 3-5 (2008)
- **C. Van Broeckhoven:** 6th International Conference on Frontotemporal Dementias, **Chair**: Session: PGRN gene/mutations**,** Rotterdam, The Netherlands, September 3-5 (2008)

Invited lectures

- **C. Van Broeckhoven:** NIH Symposium on Progranulin and the TDP-43opathies: 'PGRN in familial and sporadic FTD', Washington DC, USA, February 4-5 (2008)
- **C. Van Broeckhoven:** GEOPD meeting 2008: 'Progranulin and neurodegenerative diseases', Trondheim, Norway, June 9-11 (2008)
- **C. Van Broeckhoven:** 12th International Congress of Parkinson's disease and Movement Disorders: 'Genetics of frontotemporal lobar degenerations', Chicago, USA, June 22-26 (2008)
- **C. Van Broeckhoven:** Seminar at the national Institute on Aging, Biomedical Research Center, laboratory of Neurosciences: 'A genetic approach to neurodegenerative dementias', Baltimore, USA, September 16 (2008)

Oral presentations

- **H. Wils:** Generation of FTLD-U mouse models: conditional Grn knockout and wild-type and mutant Htdp-43 overexpressing mice. 6th International Conference on Frontotemporal Dementias, Rotterdam, the Netherlands, September 3-5 (2008)
- **K. Sleegers**: PGRN mutations in AD, ALS and PD. 6th International Conference on Frontotemporal Dementias, Rotterdam, the Netherlands, September 3-5 (2008)

Poster presentations

- **G. Kleinberger:** Progranulin (GRN) is directly involved in cell proliferation but not in caspase-activation in glial and non-glial cells. 11th International Conference on Alzheimer's disease (ICAD), Chicago, Illinois, USA, July 26-31 (2008)
- **G. Kleinberger:** Progranulin has growth modulatory property but is not directly involved in caspase-mediated apoptosis in glial and non-glial cell culture. 6th International Conference on Frontotemporal Dementias, Rotterdam, the Netherlands, September 3-5 (2008)
- **H. Wils:** Neuropathological and biochemical characterization of GRN mutation carriers and GRN conditional mice. 11th International Conference on Alzheimer's disease (ICAD), Chicago, Illinois, USA, July 26-31 (2008)
- **N. Brouwers:** Genetic variability at the progranulin locus contributes to risk for clinically diagnosed Alzheimer disease. Ipsen Foundation - Intracellular traffic and neurodegenerative disorders, Paris, France, April 28 (2008)
- **N. Brouwers:** Variability at the progranulin locus contributes to risk for Alzheimer disease. XX International Congress of Genetics, Berlin, Germany, July 12-18 (2008)
- **N. Brouwers:** Genetic variability at the progranulin locus contributes to risk for Alzheimer's disease. 11th International Conference on Alzheimer's disease (ICAD), Chicago, Illinois, USA, July 26-31 (2008)
- **N. Brouwers:** Genetic variability at the progranulin locus contributes to risk for clinically diagnosed Alzheimer disease. 6th International Conference on Frontotemporal Dementias, Rotterdam, the Netherlands, September 3-5 (2008)

Progress Report of the Research Group of

Prof. dr. F. Van Roy

Universiteit Gent (UGent)

Prof. dr. Frans VAN ROY, Ph.D.,

Department DirectorDepartment for Molecular Biomedical Research (DMBR)
VIB - Ghent University'Fiers-Schell-Van Montagu' building
Technologiepark 927
B-9052 Ghent (Zwijnaarde), Belgium
Tel: +32 9 33 13 600/13 601
Fax: +32 9 33 13 609/13 500
F.Vanroy@dmbr.VIB-UGent.be
www.dmbr.UGent.be

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) supported by the GSKE grant 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 new NBG 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 E-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. At the moment, our 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

In polarized epithelial cells, the adherens junction (AJ), which is a major type of cell–cell junctions, develops near the apical surface. It is organized as a molecular belt, in connection with a bundle of cortical actin filaments. The AJ comprises classic cadherins, catenins, and other associated proteins. Classic cadherins interact homophilically via their extracellular domain, functioning as a physical linker between the adjacent cell membranes. The cytoplasmic regions of these cadherins bind α-catenin, which in turn associates with **α-catenin**. Alpha-catenin is indispensable for cadherin-mediated cell adhesion. In its absence, the AJ is disrupted, and the apical actin belt dissociates from the cadherin–catenin complex.

As cell-cell contacts mature, the local concentration of the cadherin-catenin complex increases above

the critical concentration for α-catenin homodimerization. It is believed that at that time alpha-catenin dissociates from the complex, to dimerize, bind and bundle actin filaments, and locally inhibit the Arp2/3 complex (Drees et al., 2005). By analogy, therefore, loss of α-catenin in human cancers could result in rampant Arp2/3-mediated actin polymerization and increased formation of lamellipodia, thereby decreasing cell-cell adhesion and increasing cell migration and invasion of tumor cells. In addition to these structural effects, loss of α-catenin in cancer cell lines and deletion of α-catenin in mouse models were found to be associated with increased proliferation, decreased apoptosis, and increased growth factor signaling (Benjamin and Nelson, 2008; Vasioukhin et al., 2001).

An additional complexity is that there are three homologous α-catenin proteins. The three known α-catenins comprise the ubiquitously expressed αE-catenin (102 kDa), neural αN-catenin, and **αT-catenin**, which we had identified as a member of the α-catenin family with a restricted expression pattern (Janssens et al., 2001). AlphaT-catenin is especially abundant in heart tissue, where it is co-expressed with αE-catenin at intercalated discs, in the peritubular myoid cells of testis, and in smaller amounts in skeletal muscle and brain. Using monoclonal 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. 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 with other researchers the possible involvement of *CTNNA3* in this disabling disease. We showed that αT-catenin can inhibit Wnt signaling and that it meets the criteria for both a positional and functional candidate for AD susceptibility (Busby et al., 2004). However, none of the single-nucleotide polymorphisms of *CTNNA3* we analyzed appeared to be strongly associated with chromosome-10-linked AD (Busby et al., 2004), though 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).

Most recently we were able to produce a mouse in which the *Ctnna3* gene, encoding αT-catenin, is knocked out. We are now studying the phenotype of mice lacking αT-catenin in great detail at the anatomical, histological, physiological and behavioral levels. This will allow us to prove or disprove whether αT-catenin plays an important role in neural tissues.

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

The aim of this part of the project is 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 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 most p120ctn-encoding exons 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 all cortical layers of the brain. The brain-confined p120ctn knock-out was confirmed at the protein level by immunohistochemistry (Fig. 1A) and western blotting (Fig. 1B). N-cadherin, α-catenin and α-catenin levels were normal as compared to control mice (Fig. 1B). These mice were viable, had normal body weights, and developed brains of normal size and overall normal anatomy. Nissl-staining revealed normal brain histology. p120ctn overexpression in various cell types leads to a dendritic branching phenotype (Reynolds et al., 1996). This reflects the ability of p120ctn to modulate the activity of the **RhoGTPases**, which are known to influence normal dendritic spine density and morphology (Anastasiadis et al., 2000). Abnormal spine morphology is seen in patients with nonsyndromic mental retardation and cognitive disorders (Govek et al., 2005). We therefore performed RhoGTPase pull-down assays on brain lysates from p120 fl/fl x Nestin-Cre mice and from control mice. p120ctn-deficient mice showed higher levels of active RhoA than control mice (Fig. 1C) whereas active Rac1 levels were similar (Fig. 1D).

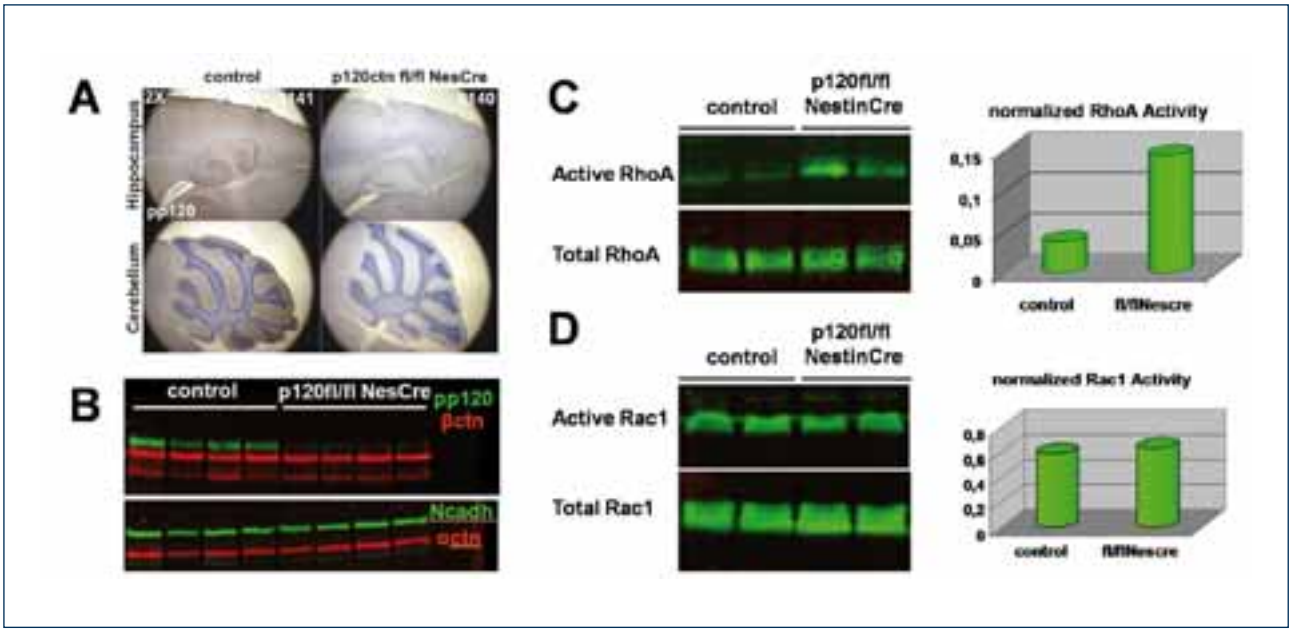

Figure 1. Analysis of control and p120ctn fl/fl x Nestin-Cre mice. (A) p120ctn immunohistochemistry in hippocampus and cerebellum. (B) Protein analysis for p120ctn, α-catenin, N-cadherin and α-catenin in brain lysates. RhoA activity (C) and Rac1 activity (D) in brain lysates of control and p120 fl/fl x Nestin-Cre mice.

To examine the influence of p120ctn exon C on normalization of this phenotype, we crossed the p120ctn fl/fl x Nestin-Cre mice with, respectively, p120 KO-C/wt and p120 KI-C/wt mice. The resultant offspring bear 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. We will analyze the phenotypes of these mice anatomically and histologically by Nissl and Golgi stainings. The preparation of hippocampal cultures from the different transgenic mice, which has been started recently, will help in the molecular and cell biological analysis. Suitable assays will be used to assess potential behavioral alterations in these 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.

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 are generating 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. 2A). This mouse will then be crossed with different Cre mice to elucidate the role of Pcdh10 in important processes, such as controlled cell proliferation, controlled migration, cell differentiation, and programmed cell death. We are also 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. This model will be used to explore the role of these conserved domains in various intracellular signaling pathways. Chimeric mice for knocking out the long isoforms are already born. In a first approach, they will be crossed with a Cre line that deletes Pcdh10 in all tissues, and the phenotype of these mice will be compared with the germline total knockout of Pcdh10.

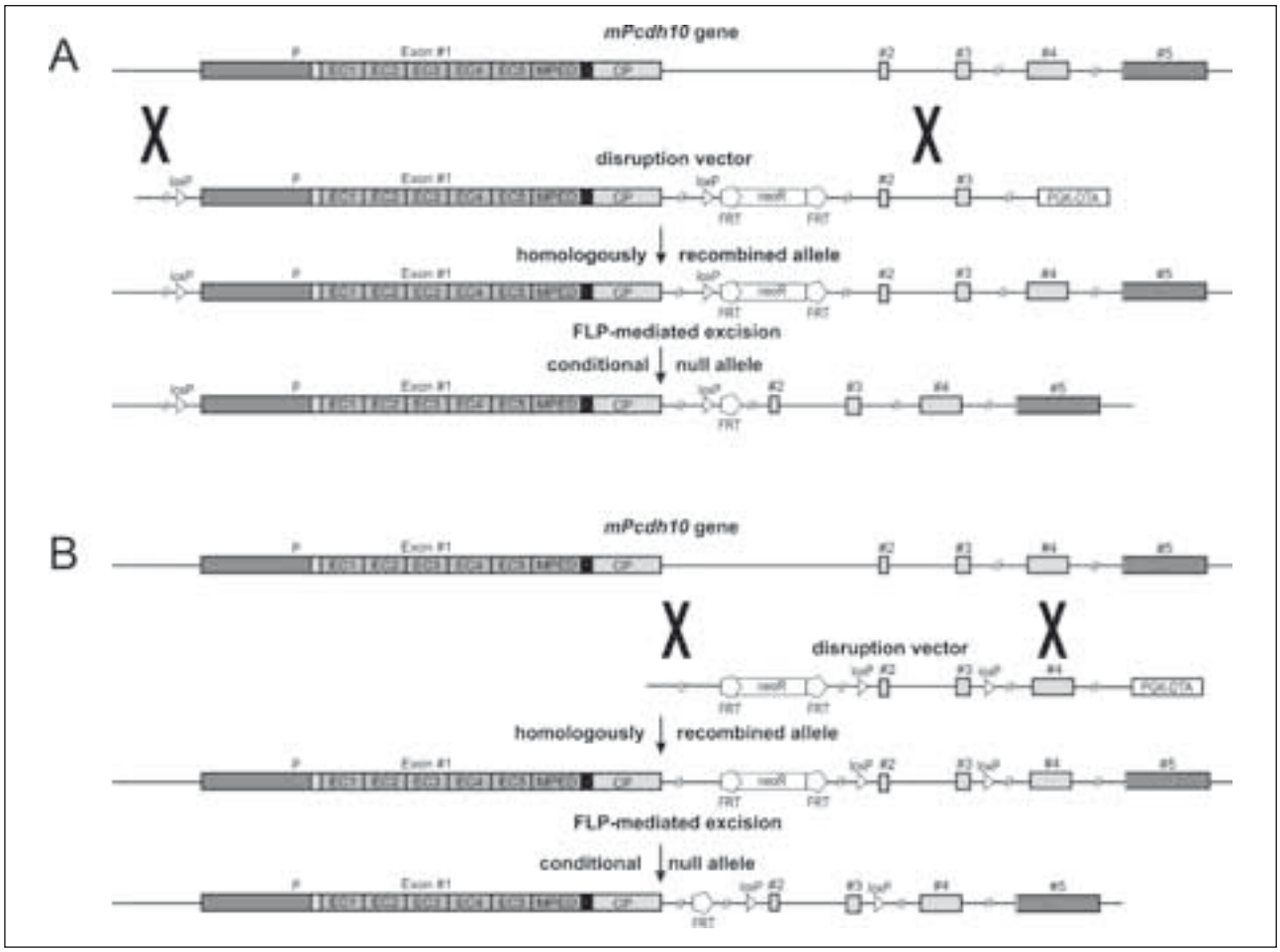

Figure 2. Construction of *Pcdh10* knockout alleles affecting either all isoforms of protocadherin-10 (A) or only the long isoform (B). Embryonic stem (ES) cells with a homologously recombined Pcdh10 allele can be selected by using a positive selection marker (neoR gene) in combination with loss of the negative selection marker (DTA, diphtheria toxin subunit A). A floxed, conditional null Pcdh10 allele is obtained upon excision of the neoR 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.

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 are 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 are currently breeding to get germline transmission of the transgene.

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) in order 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. The interaction with

PCDH11Y and cross-interactions with PCDH10 and other δ-PCDHs are being analyzed as well. The results are promising and the interactions seem to be isoform-specific. In addition, GST-pulldown experiments are ongoing for PCDH11X/Y and PCDH10 using mouse brain lysates to identify neuronal interaction partners of these protocadherins.

Functional analysis of the interactions *in vitro* will involve overexpression and knockdown (shRNA) experiments, which are routinely done in our department, followed by assays of cell aggregation, analysis of neurite extension, evaluation of dendritic spine mobility, etc.

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) found 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 the chest cavity. NBs frequently have aberrations of the chromosomal regions 1p36 and 17q11, which are involved in the translocation 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.

Expression analysis with an antibody against the NBPF proteins has shown that they are expressed in **neurons** but not in glial cells (Popesco et al., 2006). We initiated a collaborative project with Dr. Pierre Vanderhaeghen (ULB, Brussels) to analyze the expression of **NBPF transcripts** in human fetal brain by RNA *in situ* hybridization. Preliminary results on fetal brain samples of different stages are quite promising. Those experiments revealed that the *NBPF* genes are expressed in specific structures in the developing human brain. Additional experiments with probes against neuronal markers will be used to identify the cell types expressing the NBPF transcripts. The expression analysis will be complemented by detection of NBPF proteins using **specific antibodies** that we have raised.

By quantitative PCR we showed a decreased level of the *NBPF* transcripts in certain types of neuroblastoma cell lines (Vandepoele et al., 2008). Due to the high sequence similarity between the different *NBPF* paralogs, it is very difficult to determine the expression levels of distinct *NBPF* genes. However, taking advantage of the more isoform-specific 5'UTR region, we were able to set up a protocol to specifically amplify the NBPF1 transcripts. Using this protocol, we showed that the *NBPF1* expression pattern is similar to the global *NBPF* expression pattern. The expression level is significantly lower in cell lines with a heterozygous deletion of the *NBPF1* locus compared to cell lines without *NBPF1* loss (Vandepoele et al., 2008). Although this might be explained by the lower gene copy number, it is striking that such downregulation was observed for only 15-20% of the genes located in this region (Janoueix-Lerosey et al., 2004), showing that additional mechanisms besides loss of heterozygosity play a role in the downregulation of some of these genes.

To investigate the cause of this decreased NPPF1 expression level, we analyzed the corresponding promoter sequence. Interestingly, the *NBPF1* promoter shows high sequence similarity to one of the

promoters of the ***EVI5*** gene (Ecotropic Viral Integration site 5). Like *NBPF1*, *EVI5* is disrupted by a constitutional translocation in a neuroblastoma patient (Roberts et al., 1998). We showed in a set of neuroblastoma cell lines that *EVI5* expression mimics the expression pattern of *NBPF*, with low *EVI5* expression in cell lines having low *NBPF* levels, and *vice versa*. Apart from its disruption in an NB-associated constitutional translocation, no correlation has been made between *EVI5* and neuroblastoma. Although the promoter sequences of *NBPF1* and *EVI5* are almost identical, and the resulting expression levels have similar trends, we recently showed that these two genes are regulated differently in a panel of neuroblastoma cells (Vandepoele et al., 2009*).

The *NBPF1* gene may play an important role in both normal and cancerous neuronal cells. As constitutive overexpression of NBPF1 turned out to be cytotoxic (our unpublished data), we are currently using **conditional expression systems** to generate viable NBPF1-expressing cell lines (Vandepoele et al., 2008). These will be tested for their ability to undergo **neuronal differentiation** upon specific stimuli, and for changed tumorigenicity. Moreover, by searching for **NBPF1-interacting proteins**, we have obtained additional evidence that NBPF1 has a role in tumors of the nervous system. For instance, the amino-terminal domain of NBPF1 interacts with the Chibby protein, which is an antagonist of the Wnt/Wingless pathway (Takemaru et al., 2003), a key pathway in neural crest development and oncogenesis. The functional implications of the NBPF1-Chibby interaction need further investigation. Other molecular NBPF1 interactions are also being investigated.

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Progress Report of the Research Group of

Dr. P. Vanderhaeghen

Université Libre de Bruxelles (ULB)

Dr. Pierre Vanderhaeghen

Institute of Interdisciplinary Research (IRIBHN)
ULB, Campus Erasme
808, Route de Lennik
B-1070 Brussels
Tel.: +32 2 555 41 86
Fax: +32 2 555 46 55
pvdhaegh@ulb.ac.be

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 2008 thanks to the Funding of the FMRE/GSKE, providing a link with recently published work, 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 lineage, expressing Pax6, Tbr2, Emx1-2, Otx1-2 and Fox-G1 transcription factors.

ES cell-derived cortical-like progenitors subsequently differentiate into a stereotyped population of neurons, most of which 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: cells expressing markers of Cajal-Retzius neurons are first being generated, follwowed by pyramidal neurons expressing markers of deep cortical layers, and eventually by neurons expressing markers of the superfical layers. Finally, ES cell-derived cortical neurons can effectively integrate into postnatal cortical tissue, display electrophysiological and synaptic activity profiles indistinguishable from excitatory cortical neurons. 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).

Our results indicate that ES cells can be efficiently specified into cortical neural progenitors and pyramidal

neurons in a chemically defined medium lacking any morphogen and containing only morphogen inhibitors, thereby surprisingly suggesting a “default pathway” of cortical neuron specification (Gaspard et al., 2008). 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 also further explore whether and how ES-derived cortical neurons can effectively function in the host brain (including in models of cortical lesions), using a combination of anatomy, physiology and functional imaging. On the other hand we are trying to implement the system to human ES cells. The ability to differentiate in vitro cortical neurons from hES cells would constitute a primary tool to study human cortical neuron development. Finally we will try to generate novel models of neurodevelopmental diseases, by generating or obtaining specific iPS cell lines from patients displaying some of these rare diseases (Takahashi and Yamanaka, 2006), and studying them using our in vitro models of corticogenesis.

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 control of another important aspect of forebrain development: neuronal migration.

Work from several laboratories has demonstrated that distinct types of forebrain neurons are generated in different dorso-ventral domains and migrate radially or tangentially to their final destination (Marin and Rubenstein, 2003). For instance GABA-ergic interneurons destined to the cerebral cortex and striatum are generated within the medial GE (MGE), while the lateral GE (LGE) essentially generates GABA-ergic projection neurons destined to the striatum. This dorso-ventral sorting of migrating neurons may have important functional and behavioural consequences, such as the balance of GABAergic innervation in the striatum and cortex. In addition, the mammalian striatum can be subdivided into two morphologically and functionally defined compartments, the matrix and the striosomes, which underlies important functional features of the striatum (Gerfen, 1989). Matrix and striosome neurons are generated sequentially during embryonic development, and segregate from each other to form a mosaic of distinct compartments (van der Kooy and Fishell, 1987). However, the molecular mechanisms underlying this time-dependent process of neuronal segregation remain largely unknown.

To gain insight into the mechanisms involved in these processes, we designed novel organotypic assays to study striatal development compartmentation. In this system, eGFP+ neurons, taken at different embryonic ages, are dissociated on top of early postnatal slices to test for their capacity to recognize the presumptive matrix and striosome compartments (Passante et al., 2008). This assay allows to

recapitulate the cardinal feature of striatal patterning, i.e. that neurons generated at different time points segregate in different striatal compartments. On the other hand, using an in situ hybridization expression screen for putative guidance factors of striatal neurons, we identified ephrin/Eph family members as candidate genes controlling temporal patterning of the striatum. Indeed, some ephrin ligands are mainly expressed in the early striosome compartment, while some Eph receptors are preferentially expressed in the late-generated matrix compartment (Passante et al., 2008). We next used the in vitro assay to test and demonstrate the functional requirement of these factors for the correct segregation of matrix vs striosome neurons. This was further confirmed in vivo by the analysis of the striatal patterning in ephrin-A5/EphA4 compound mutant mice, which revealed that ephrin-A5/EphA4 genes are required for the proper sorting of matrix and striosome neuronal populations in vivo. These data constitute the first identification of guidance genes involved in the control of the patterning of the striatum, supporting a novel model whereby the temporal control of neuronal guidance cues enables the development of the specific cytoarchitecture of this structure (Passante et al., 2008).

In parallel we have set up organotypic assays to dissect the molecular and cellular mechanisms that control the migration of distinct populations of neurons to dorsal vs ventral domains of the telencephalon: in this system, GE explants overexpressing eGFP (obtained from transgenic mice or following focal electroporation) are co-cultured with organotypic slices of developing telencephalon and the migration patterns of eGFP+ cells can be monitored over several days. These assays allow to recapitulate several important aspects of tangential migration, such as the dorso-ventral sorting of MGE vs LGE neurons that occurs in vivo. Using these in vitro assays, we have 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 in vitro observations using in utero electroporation and appropriate mouse transgenic models (in particular ephrin-B1-2 conditional kos, available in the laboratory), in order to test for the consequences of the early disruption of migration patterns on cortical and striatal function in mature animals.

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 recently 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 have just been generated. In parallel we generate knock-out mice for the mouse HAR1 gene.

Using a similar approach combining computational biology and expression analyses centered on the

developing human brain, we also identified the expression in the human embryonic brain of several other uhman-specific genes, including a novel human-specific form of the CDC14 phosphatase, in collaboration with the group of H. Kaessmann (U. Lausanne), (Rosso et al., 2008).

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 (Lambot and PV, unpublished data). The most promizing candidates will be studied functionally in the mouse and in ES cell-based models of cortical development.

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Publications of the laboratory in 2008 in the frame of the FMRE/GSKE grant:

- An intrinsic mechanism of corticogenesis from embryonic stem cells.
  Gaspard,N., Bouschet,T., Hourez,R., Dimidschstein,J., Naeije,G., van den Ameele J., Espuny-Camacho,I., Herpoel,A., Passante,L., Schiffmann,S.N., Gaillard,A., and **Vanderhaeghen,P.**
  Nature (Article) 2008 455, 351-357.
- Temporal regulation of ephrin/Eph signalling is required for the spatial patterning of the mammalian striatum.
  Passante,L., Gaspard,N., Degraeve,M., Frisen,J., Kullander,K., De,M., V, and **Vanderhaeghen,P.**
  Development 2008 135, 3281-3290.
- Birth and rapid subcellular adaptation of a hominoid-specific CDC14 protein. Rosso,L., Marques,A.C., Weier,M., Lambert,N., Lambot,M.A., **Vanderhaeghen,P.**, and Kaessmann,H.
  PLoS. Biology 2008 6, e140.

Progress Report of the Research Group of

Prof dr. W. Vanduffel

Katholieke Universiteit Leuven (K.U.Leuven)

Prof. Dr. Wim Vanduffel

Lab. Neuro- en psychofysiologie
Fac. Geneeskunde, K.U. LEUVEN
Herestraat 49
B-3000 Leuven
Tel.: +32 16 34 57 40
Fax: +32 16 34 59 93
E-mail: wim.vanduffel@med.kuleuven.ac.be

Collaborators:

Leeland Ekstrom, John Arsenault, Pieter Roelfsema, Giorgio Bonmassar, Hauke Kolster

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

In 2008, we published the first study in which we combined intracranial microstimulation and fMRI in awake behaving monkeys (Ekstrom et al., 2008). We showed that the combination of these two techniques is an exquisite tool to perform in-vivo tractography experiments. Moreover, we described that artificially increased output of the Frontal Eye Field of the monkey modulates activity in visual cortex in a spatially heterogeneous manner. Also, presumptive feedback signals from this frontal structure modulates activity in early visual areas not directly connected with the FEF only when a retinal stimulus is present. This indicates that bottom-up input is needed to gate feedback signals towards the earliest stages of the visual processing stream. As will be outlined below, another study revealed that the type of stimulus, in addition to its spatial location with respect to the stimulated FEF movement fields, is critical for the FEF-dependent modulation of activity in visual cortex. In particular we show that modulatory effects are the strongest for less salient stimuli.

Importantly, the new Siemens 3T scanner for experimental animal research is now fully operational. A Hercules grant was recently awarded which will enable us to expand the MRI system with the strongest available gradient systems for a clinical scanner (i.e. the AC88). These gradients will be installed in 2009. The addition of the AC88 will allow us to considerably improve the spatial resolution of our measurements while retaining the signal to noise ratio and reducing distortions between the functional and anatomical images.

Modulation of the Contrast Response Function by Electrical Microstimulation of the Macaque Frontal Eye Field

Spatial attention influences representations in visual cortical areas as well as perception. Some models predict a contrast gain, while others a response or activity gain when attention is directed to an isolated, contrast varying stimulus. Recent evidence has indicated that microstimulating the Frontal Eye Field (FEF) can modulate V4 neuronal firing rates that resemble spatial attention-like effects, and we have shown similar modulations of functional magnetic resonance imaging (fMRI) activity throughout the visual system. In the present study, we used fMRI in awake, fixating monkeys to first measure the response in twelve visual cortical areas to stimuli of varying luminance contrast. We simultaneously microstimulated sub-regions of the FEF with movement fields that overlapped the stimulus locations and measured how this artificially increased FEF output modulated contrast response functions (CRFs) throughout visual cortex. In general, we found evidence for a non-proportional scaling of the CRF under these conditions, 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. Further, we measured a characteristic spatial pattern of enhancement and suppression across the cortical surface, from which we propose a simple schematic of this contrast-dependent fMRI response.

Results

In two monkeys, we collected data associated with four stimulus locations, corresponding to four implanted FEF electrodes and associated movement fields (MFs). Contrast-varying stimuli (Figure 1) were spread across the left visual field, contralateral to the side of the brain in which we implanted the electrodes, with examples in the upper and lower visual field and along the horizontal meridian.

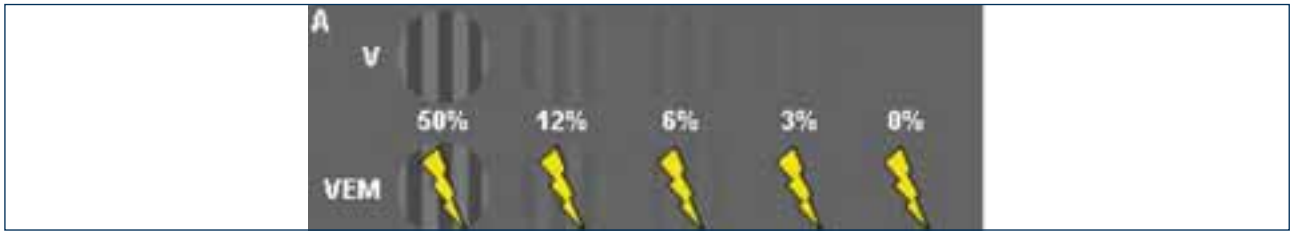

Figure 1 Luminance contrast varying stimuli presented in the FEF-movement fields

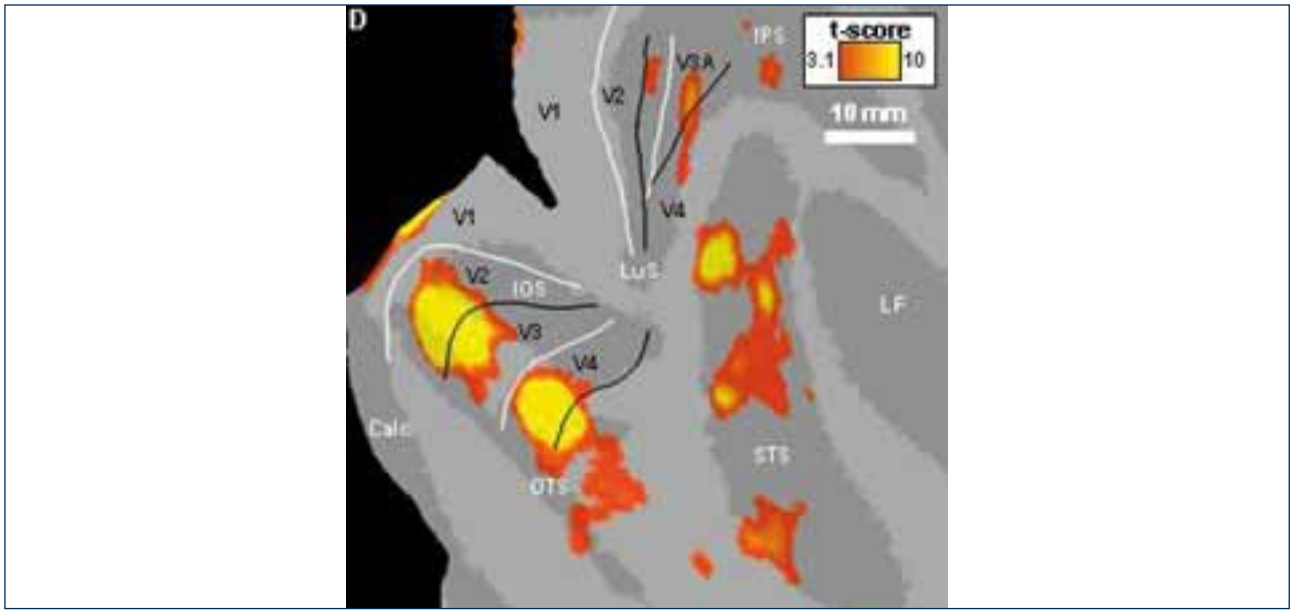

Figure 2. Visually-driven activity by high-contrast stimulus used to define the ROI's

Visual-only Contrast Response Functions

For each stimulus location, we identified a population of *visual voxels* for further analysis from the fMRI contrast defined by the main visual effect at the highest luminance contrast (Figure 2). Using this population of voxels, we extracted the percent change in MR signal with respect to the fixation only or 0% V condition for each voxel at each of the other luminance contrast levels presented to the subjects. For each cortical area of interest, we then pooled the population of *visual voxels* across all sessions from both subjects and computed the mean response as a function of contrast to generate a V CRF.

Contrast Response Functions with FEF-EM

We also extracted the percent change in MR signal for the epochs that combined a visual stimulus with simultaneous electrical stimulation of the FEF location with a matching movement field (i.e. the VEM condition). After comparing the difference between the VEM and V epochs, we also computed the fractional change this difference represents in terms of the underlying visual-only activation. Figure 3 shows this fractional change as a function of contrast for a selection of visual areas.

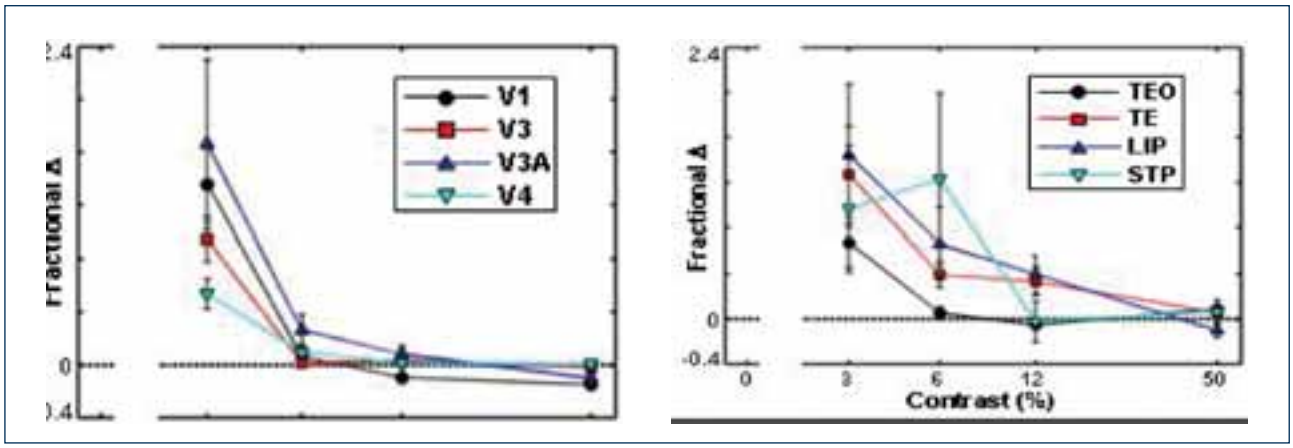

Figure 3. Modulation of the contrast response function in several visal areas by microstimulation of the FEF. Fractional change in percent MR signal, (VEM-V)/V are plotted indicating the effective change in fMRI activity caused by FEF-EM relative to the visual-only activation level.

In all areas except STP, we observed the largest increase in fMRI activity in the VEM epochs at the 3% luminance contrast level, while the highest contrast stimuli showed no or even a negative interaction, which indicates a reduction in fMRI activity due to EM. We think these high contrast observations are not likely attributable to a ceiling effect of our fMRI measurements, given that we found a decrease in activation in some regions and were able to measure much larger percent signal change values in some areas in our previous study.

Generalized CRF for Visual Cortex

By pooling all voxels from the identified cortical areas, we calculated an overall CRF, difference CRF and fractional change function for all visually driven voxels in occipital cortex. We observed a significant positive interaction at 3% luminance contrast and a significant negative interaction at 50% luminance contrast. In addition, we measured a significant overall baseline shift at 0% contrast ($P < 0.05$; one sample, two tailed *t*-test). In an additional analysis, we split our population of *visual voxels* into highly and weakly visually responsive groups, on the basis of each voxel's *t*-score in the localizer contrast (that is, 50% VEM + 50% V versus 0% VEM + 0% V). All voxels that exceeded a *P* value of 0.05 at the corrected level were included in the *high* population (in other words, visually very well driven voxels). The remaining visual voxels (that is: 0.05, corrected < $P < 0.05$, uncorrected) were included in the *low* population. Both the weakly and strongly driven voxels exhibited a baseline shift, or an increase in activity due to FEF-EM in the absence of visual stimulation ($P < 0.05$; one sample, two tailed *t*-test). Further, we observed a remarkable difference in the effects of FEF-EM on the CRF of the *low* and *high* population of voxels. The *low* population displayed a significant positive interaction at 3% and 12%, and a significant negative interaction at 50% ($P < 0.05$; two-way ANOVA). For the *high* population, we also found a positive interaction at 3%; however, we found a negative interaction at all three higher contrast levels ($P < 0.05$; two-way ANOVA).

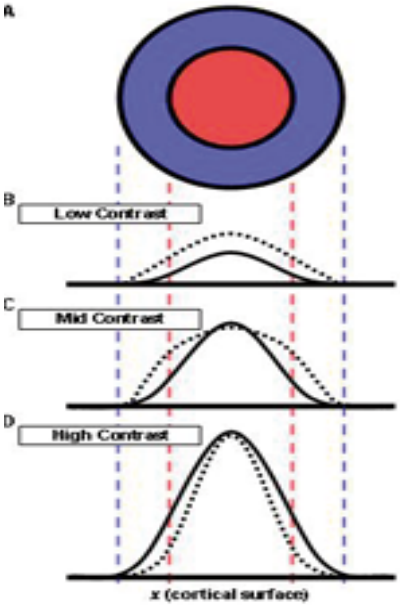

Figure 4 Schematic summary of the effect of FEF-EM. *A*, The concentric circles correspond to regions on the two-dimensional cortical surface representation of a visual stimulus. Red / blue match the well / less well driven populations of *visual voxels*. Projecting this surface to one dimension yields three different activity profiles, depending on the stimulus contrast used (solid curve represents visual-only activation, dotted curve the combined response to visual stimulation and FEF-EM).

The results of the split-population analysis are summarized schematically in Figure 4. The colored circles represent the two populations of voxels on the cortical surface, with the highly responsive voxels in the center and the weakly responsive voxels in the margin of the schematic. The lower panels of Figure 4 show a projection of the activity profile onto a single spatial dimension. For the lowest contrast stimuli, FEF-EM amplified the activity of both weakly and strongly driven voxels (compare solid, V-response curve and dotted, VEM-response curve, Fig. 4*B*). For the highest contrast stimuli (Fig. 4*D*), the macroscopic circuit switched to a discriminatory mode; the activity of all voxels was slightly suppressed, though weakly driven voxels were suppressed more. For intermediate contrasts (Fig. 4*C*), the results show a mixture of effects: the activity of weakly responsive voxels were still amplified, while strongly responsive voxels were slightly suppressed.

Discussion

Using CBV-weighted fMRI, we have measured response functions to visual stimuli at five luminance contrast levels in twelve defined functional areas of the macaque visual cortex. Further, we observed how this contrast response was modulated by the simultaneous stimulation of FEF sites with movement fields at the location of the visual stimuli. We observed a significant baseline shift (i.e. at 0% contrast) due to FEF-EM in a subset of areas known to be well connected to the FEF, and in general we found the largest increases of fMRI activity for the lowest contrast stimuli that produce smallest amounts of visual drive in cortical areas. High contrast stimuli, which produce the largest drive, were in general unaffected or reduced by FEF-EM.

Modulation of CRFs by FEF-EM

The models that have been proposed to describe the effects of attention on neural activity in the visual cortex (*1*) provide a useful framework for describing the effects of stimulating the FEF. One model that has been proposed for the effects of attention on CRFs is the response gain model, which suggests that the effect of attention is equivalent to a proportional increase in response at all contrasts so that the effect is most pronounced at the highest contrasts. Under the conditions of our study, we did not observe evidence for a response gain model; instead, in many areas and in the overall response across visual cortex we saw a reduction in activity at high contrast. An alternative model is the contrast gain model, which proposes that the effect of attention is an increase in the apparent contrast of a stimulus, causing a leftward shift of the entire CRF (*2*).

The principal characteristic of a contrast gain effect is an increase in activity for stimuli with intermediate contrasts, with little effect at baseline and saturation activity levels. We do observe common features with this model, specifically at lower and intermediate contrasts. The correspondence is not perfect, however, particularly at high contrast.

As we noted above, the previous studies that investigated the effects of attention on CRFs compared an attend-toward to an attend-away condition in the presence of multiple stimuli. Activity in an attend-away condition is presumably weaker than activity in the no-EM condition that we used as a baseline, for two reasons. First, the simultaneous presentation of multiple stimuli likely yielded suppressive interactions between them (REF), which were not present in our study using only a single stimulus. Second, withdrawal of attention from non-attended locations may have further suppressed the response in these previous studies. Thus, the previous measurements that supported a proportional gain model of attention, by showing a larger enhancement of neuronal activity at higher contrasts, were made relative to a potentially weaker baseline response than our no-EM baseline condition. However, we cannot exclude the alternative explanation that FEF-EM does not reproduce all effects of selective attention on neuronal activity in the visual cortex.

Our results, in particular the schematic of Fig. 4, bear resemblance to previous frameworks for center-surround interactions in rat somatosensory cortex (*3-6*) and models for contrast-dependent interactions in area V1 (*7, 8*). We suggest that the presumptive feedback connections that are activated from area FEF: (i) amplify all the input at low levels of visual drive, (ii) sharpen salient responses at high levels of activity (i.e. increase discriminability), and (iii) cause a mixture of these effects at intermediate activity levels. This mixture of effects is consistent with our previous findings (*9*), where we used chromatic stimuli with approximately 31% luminance contrast and observed a slight suppression of the fMRI responses at the center of the representation of these stimuli that was accompanied by an enhancement of activity in the surrounding voxels that were less well activated. At first sight, these findings may appear to be at odds with a previous report of FEF-EM causing the largest enhancement of V4 neuronal firing rates for stimuli with an optimal orientation (*10*). We note, however, that the use of fMRI precludes the stimulation of all neurons within a voxel with the optimal stimulus orientation so that the apparent discrepancy may be due to the combination of responses of neurons with a range of stimulus preferences.

Our results strengthen theories proposing that increased output from areas responsible for oculomotor control modulates the incoming visual activity (*10-14*). Moreover, we have found that this top-down modulation depends on the underlying sensory drive so that neuronal activity in the visual cortex is amplified at the lower stimulus contrasts, but sharpened at higher contrast levels.

Papers 2008

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Progress Report of the Research Group of

Prof dr. R. Vogels

Katholieke Universiteit Leuven (K.U.Leuven)

Prof. dr. Rufin Vogels

Laboratorium voor Neuro- en Psychofysiologie
K.U. Leuven Medical School.
Campus Gasthuisberg
Herestraat 49
3000 Leuven
Tel.: +32 16 34 58 39
Fax: +32 16 34 59 93
rufin.vogels@med.kuleuven.ac.be

Coding of biological motion in macaque monkeys: relating perception and neuronal selectivity.

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.

In our previous study, we have studied the coding by macaque temporal cortical neurons of a parameterized set of dynamic images of simple visual actions. We used arm 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 indicate that rostral STS neurons respond selectively to temporal segments of the action movies, but not to the whole action as such (Vangeneugden, Pollick and Vogels, *Cerebral Cortex*, in press). Nonetheless, the population of STS neurons was able to represent the similarity between the action movies. 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 and most of these responded less to the moving arm presented in isolation than to whole body configuration. 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. Most of these "motion" neurons responded as well to the whole body configuration as to the moving arm alone. In fact, further testing showed that most of the "motion" neurons responded at least equally well to the motion trajectory of the wrist point presented alone. These selective responses to the end-effector are compatible with action coding since most of the information about these arm actions is present in the motion pattern of the end-effector.

In the above described experiments we used simple arm movements in which most of the motion information was present in one point (the wrist). In the next series of studies, we are using more complex motion patterns, i.e. those of a walking and running human. These more complex motion patterns 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 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) and they created different types of stimuli using these data: shaded cylinders connecting the joints ("humanoids"), point-light displays and stick figures. Unlike in previous neurophysiological studies, 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.

We have trained 3 rhesus monkeys to discriminate whether the walking is forward (rightward) or backward (leftward- with the body oriented to the right) and whether the actor is walking forward to the left versus forward to the right. Each of the 3 animals learned the latter left versus right walking relatively fast, which is expected since both motion and snapshot form information differs between walking directions. Since (naive) humans find it extremely easy (100% correct) to discriminate forward from backward locomotion,

it was very surprising to us that it took several months of intensive training in each of the 3 animals to learn the animals forward versus backward locomotion. In the case of this forward versus backward walking, the snapshots are identical and only differ in their temporal sequence - in fact the backward movies are the forward movies played in reverse -. Thus, discrimination of forward versus backward walking can only be done by taking into account temporal sequence information and it turns out that monkeys do not seem to use such information as spontaneously as we humans do (but can be trained to do so).

We trained first the animals in the discrimination of direction of a walking "humanoid" at an intermediate speed (walking speed of 4.2 km/h) and after successful training, we tested for generalization to (1) walking of the same individual at different speeds, (2) walking of different individuals (with different walking "style"), (3) running at different speeds of the same individual, and (4) point light displays of the same actions. These displays were presented with a low frequency together with the familiar, trained 4.2 km/h displays. The degree of generalization varied among the 3 animals but the following tendencies were present: regarding forward versus backward locomotion, there was good generalization to different walking speeds and actors but less so from walking to running. We found no transfer from the trained discrimination of locomotion direction of the humanoid displays to those of point light displays. To our knowledge, the perception of biological motion in point light displays has not been demonstrated behaviorally in nonhuman primates. Our data suggest that macaques might perceive no actions but instead a meaningless bunch of moving points in point light displays. In addition, we also showed that for forward-backward motion the animals use mainly information present in the lower limbs but not in the upper limbs.

We have recorded the responses of single temporal cortical neurons to the "humanoid" locomotion stimuli in two of these extensively trained animals. In our first examination of the neuronal responses to locomotion stimuli, we tested the responses during passive fixation of the stimulus displays. This allowed us to test the responses and selectivity of the neurons to a wider range of stimulus conditions than for which the animals were trained. Thus far we have not seen clear responses to the locomotion displays in the rostral dorsal bank of the STS (area STPa; at the anterior-posterior level of area TE). However, we have found responsive neurons in the ventral bank of the STS and the lateral convexity of inferior temporal cortex (IT; area TE). The properties of the neurons were examined in different tests. In a first test, we measured whether the neurons show selectivity for walking direction (8 directions; 45 deg difference) and whether they can distinguish forward versus backward walking. Many of the thus far recorded neurons (N= 50) showed tuning for walking direction. According to a preliminary analysis, significant selectivity for backward versus forward walking was only present in a few neurons. The locomotion displays for different walking directions differ in their snapshots (the view/orientation of the walker differs between directions) and kinematics, while forward and backward locmotion differs only in kinematics (temporal sequence but not snapshots differs). The overall weak selectivity for forward versus backward walking suggests that lower bank STS neurons are not very sensitive to the temporal sequence information; however, recordings in other responsive STS patches need to be performed to substantiate this suggestion. In a second test, we compared the responses to static presentations of snapshots of the locomotion movies to those to the locomotion movies. We found that the large majority of the ventral bank STS neurons responded strongly to the static snapshots presentations. These ventral bank STS neurons can contribute to action coding by coding for walking postures – similarly to the form pathway in the Giese and Poggio model (*Nature Review Neuroscience*, 2003) of action recognition -. This would require however that the neurons show a selectivity for posture, i.e. respond stronger to some than to other snapshots of the walker. That was indeed what we found: several of

the lower bank STS neurons responded strongly to only a few of the 7 tested snapshots of a movie. Given the importance of the walking versus the running difference in the generalization behaviour of the monkeys (see above) we compared in a third test the responses of the neurons to different walking and running speeds. Some of the neurons responded differently to the different walking and running speeds.

In 2009 we will continue the single cell recordings in temporal visual cortex, in particular in other patches of the lower and upper bank STS and will perform an in-depth analysis of the data – especially on the responses to forward versus backward motion. Also, we will compare neurometric and psychometric thresholds with spatiotemporal scrambled displays of walking direction. The animals have already been trained for the scrambled displays.

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 a second series of studies, we have examined the stimulus selectivity of this adaptation effect in macaque temporal cortex. Sawamura, Orban and Vogels (*Neuron*, 2006) tested in a single-cell study in inferior temporal cortex whether the stimulus selectivity of the neuronal responses and the stimulus selectivity of the adaptation effect matches. They found much smaller adaptation when two highly dissimilar, but equally effective images were presented compared to the repetition of the same image, suggesting stimulus-specific adaptation. In the present study, we used 4 parametric sets of 6 shapes (each created by morphing between 2 complex 3D shapes) instead of one test stimulus as in Sawamura et al (2006). Sequences of 2 stimuli (stimulus durations 300ms, ISI 300ms) were presented during passive fixation or when the animals were performing a difficult luminance change detection task. Stimulus sequences included all possible 36 stimulus combinations (fully-crossed design) of the set for which the neuron was most selective. This allowed the assessment of the effect of adaptation on the responses of IT neurons as a function of the similarity between the adapter and the test stimulus. We measured simultaneously, using the same electrode, single-cell spiking activity and local field potentials (LFPs). Additionally, we examined to which degree the stimulus effectiveness of the adapter affects adaptation. We found consistent adaptation of spiking activity and LFP power in high- (gamma) but not low-frequency bands when repeating a shape. This was true when the animals were passively fixating as well as when they were performing a detection of a slight dimming of the stimuli (equating attention for first and second stimulus presentations). The degree of similarity between adapter and test stimulus features was a stronger determinant of the adaptation than was the response to the adapter for both spiking activity and gamma power. This was the case for both differences in shape and position between adapter and test stimulus. The adaptation was 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). These data demonstrate that adaptation of IT spiking and LFP activity depends on suppression of or at the input to the neurons.

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Geneeskundige Stichting Koningin Elisabeth – G.S.K.E.
Fondation Médicale Reine Elisabeth – F.M.R.E.
Queen Elisabeth Medical Foundation – Q.E.M.F.

Mailing address:

The scientifique director:
Prof. dr. em. Baron de Barsy
3, avenue J.J. Crocq laan
1020 Bruxelles - Brussel
Belgium
Tel.: +32 2 478 35 56
Fax: +32 2 478 24 13
E-mail: thierry@debarsy.be

and

The Secretary:
Mr. Erik Dhondt
3, avenue J.J. Crocq laan
1020 Bruxelles - Brussel
Belgium
Tel.: +32 2 478 35 56
Fax: +32 2 478 24 13
E-mail: fmre.gske@skynet.be
E-mail: e.l.dhondt@skynet.be
www.fmre-gske.be
www.fmre-gske.eu