Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
Königin-Elisabeth-Stiftung für Medizin
Queen Elisabeth Medical Foundation

# Verslag – Rapport – Bericht – Report

## 2012

**G.S.K.E. – F.M.R.E. – K.E.S.M. – Q.E.M.F.**

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# Geneeskundige Stichting Koningin Elisabeth
# 2012
# Inleiding verslag activiteiten van de GSKE – FMRE

Het jaar 2012 is zonder bijzondere problemen verlopen ondanks de vrees voor financiële moeilijkheden te wijten aan de internationale economische toestand.
De Raad van Bestuur heeft eind 2011 beslist om de kredieten voor 2012 te behouden en heeft deze beslissing ook bevestigd voor 2013.
Dit nieuws werd met enthousiasme en erkentelijkheid onthaald door de gesubsidieerde onderzoeksploegen.
Dus er zal opnieuw 520.000 euro verdeeld worden tussen de 13 geselecteerde ploegen in 2013.

De jaarlijkse prijsuitreiking vond plaats op 15 mei in het Koninklijk Paleis in aanwezigheid van Prinses Astrid en de hoge dignitarissen van het Paleis. De laureaten werden geselecteerd door het Wetenschappelijk Comité op basis van hun activiteiten verslag van 2011.
Volgende prijzen werden uitgereikt: prijs Burggravin Valine de Spoelberch aan de professoren Vincent Timmerman en Peter De Jonghe van de UA, de Solvay Prize aan professor Marc Cruts van de UA, de prijs Baron van Gysel de Meise aan de professoren Ilse Smolders en Ann Massie van de VUB, de ING prijs aan Professor Claudia Bagni van de KU Leuven, de prijs Janine en Jacques Delruelle aan de dokters Laurent Nguyen en Brigitte Malgrange van de ULg.

De plenaire lezing werd gegeven door professor Richard Zimmermann over de vooruitgang bij de diagnose van de degeneratieve ziekten van het centraal zenuwstelsel en in het bijzonder de ziekte van Alzheimer.
Deze presentatie werd zeer gewaardeerd door de toehoorders niet alleen omwille van de kwaliteit van de spreker maar ook omwille van de positieve toekomstvisie.

Op 27 september bracht Prinses Astrid, vergezeld van Barones Delruelle en enkele leden van de Geneeskundige Stichting Koningin Elisabeth, een bezoek aan de laboratoria van de dokters Laurent Nguyen en Brigitte Malgrange, onderzoekers en laureaten van de ‘’prijs Janine en Jacques Delruelle ’’, van de ULg.
Professor Gustave Moonen, hoofd van de dienst Neurologie en verantwoordelijke van het departement, gaf bij deze gelegenheid een historisch overzicht van de tussenkomst van de Stichting in de werking van zijn laboratorium. Hij onderstreepte ook het grote belang van deze steun voor de ontwikkeling van het neuro wetenschappelijk onderzoek.
Hij betuigde eveneens zijn erkentelijkheid aan Prinses Astrid voor de voorname rol die Ze vervult in de Stichting door Haar verenigende en dynamiserende persoonlijkheid.

Dit jaarlijks verslag 2012 is ook een gelegenheid om de gulle schenkers opnieuw van harte te danken. Zij hebben ervoor gekozen om het neuro wetenschappelijk onderzoek van de teams in onze universiteiten te steunen met een belangrijke materiële bijdrage, hetzij individueel, hetzij in de naam van een bedrijf. Hiervoor zijn wij hen allen zeer dankbaar.

Onze dank gaat ook naar Prinses Astrid, onze erevoorzitster, de leden van het Wetenschappelijke Comité, de leden van Raad van Bestuur en de administratieve medewerkers, die bereid zijn om veel tijd te besteden in het kader van hun bevoegdheid en zo bij te dragen tot een goede werking de Geneeskundige Stichting Koningin Elisabeth.

Prof. em. dr. Baron de Barsy,
wetenschappelijk directeur
Brussel, december 2012

# Fondation Médicale Reine Elisabeth
# 2012
# Introduction rapport d'activités de la FMRE - GSKE

L'année 2012 s'est passée sans problèmes particuliers malgré la crainte de difficultés financières dues à la situation économique internationale.
Le conseil d'administration avait décidé fin 2011 le maintien des crédits alloués pour 2012 et a pu confirmer cette décision pour l'année 2013.
La nouvelle fut accueillie avec enthousiasme et reconnaissance par les équipes de recherches subventionnées. Ce sera donc à nouveau 520.000 euros qui seront répartis parmi les 13 équipes sélectionnées pour l'année 2013.

Le 15 mai a eu lieu au Palais Royal en présence de la Princesse Astrid et des hauts dignitaires du Palais, la remise annuelle des prix aux lauréats sélectionnés par le comité scientifique sur la qualité de leur rapport d'activité de l'année 2011, il s'agit du prix Vicomtesse Valine de Spoelberch attribué aux professeurs Vincent Timmerman et Peter De Jonghe de l'UA, du Solvay Prize attribué au professeur Marc Cruts de l'UA, du prix Baron van Gysel de Meise attribué aux professeurs Ilse Smolders et Ann Massie de la VUB, du prix ING attribué au professeur Claudia Bagni de la KU Leuven et du prix Janine et Jacques Delruelle attribué aux docteurs. Laurent Nguyen et Brigitte Malgrange de l'ULg.

La conférence plénière était donnée par le Professeur Richard Zimmermann, sur les progrès dans le diagnostic des maladies dégénératives du système nerveux central et plus particulièrement dans la maladie d'Alzheimer.
Cet exposé a été fort apprécié par l'assemblée, pour la qualité de l'orateur d'une part mais aussi pour les espoirs qu'il laissait entrevoir pour l'avenir.

Le 27 septembre, la Princesse Astrid, accompagnée de la Baronne Delruelle et de quelques membres de la Fondation Médicale Reine Elisabeth, a visité à l'Université de Liège, le laboratoire des chercheurs Nguyen et Malgrange, lauréats du prix ''Janine et Jacques Delruelle''.
Le Professeur Gustave Moonen, responsable de la chaire de neurologie et responsable du département, a ,à cette occasion, présenté un aperçu historique de l'intervention de la Fondation dans l'activité de son laboratoire et souligné combien l'appui de la Fondation est fondamentale dans le développement des recherches en neurosciences.
Il a également exprimé toute sa reconnaissance à la Princesse Astrid pour le rôle capital qu'elle joue dans la Fondation grâce à sa personnalité fédératrice et dynamisante.

Ce rapport annuel 2012 me permet de renouveler tous mes remerciements aux généreux mécènes qui ont choisi d'aider dans nos universités les chercheurs en neuroscience par une contribution matérielle conséquente, soit à titre individuel, soit au nom d'une société. Nous leur en sommes tous très reconnaissants.

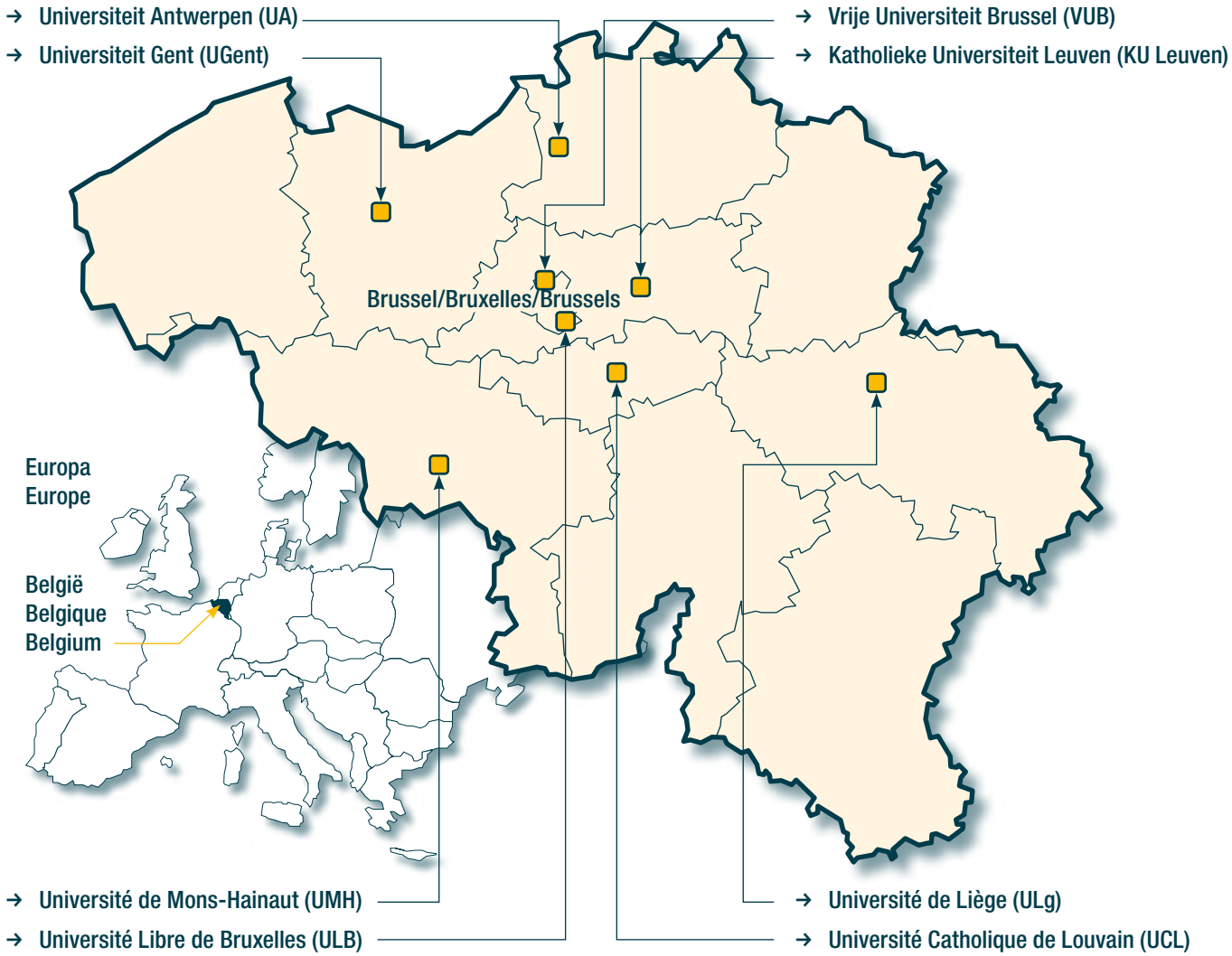
Nos remerciements vont également à la Princesse Astrid, notre présidente d'honneur, aux membres du conseil scientifique, aux membres du conseil d'administration et aux collaborateurs administratifs, qui acceptent de consacrer beaucoup de temps dans le cadre de leur compétence, au bon développement de la Fondation Médicale Reine Elisabeth.

Prof. em. dr. Baron de Barsy,
directeur scientifique
Bruxelles, décembre 2012

# 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 2011-2013

# Programmes de recherche subventionnés par la F.M.R.E. - Programme 2011-2013

# Q.E.M.F. funded research projects - Program 2011-2013

## KU Leuven

- **Prof. dr. Claudia Bagni**
  *mRNA metabolism at synapses and spine remodeling: insights into fragile X, autism and Schizophrenia.*

- **Prof. Danny Huylebroeck, Phd**
  *Developmental origin of multiple defects of the nervous systems in Mowat-Wilson syndrome and its new insights for normal embryonic and adult neurogenesis.*

## UA

- **Prof. dr. Marc Cruts, Phd**
  *Molecular genetics and functional genomics of frontotemporal lobar degeneration.*

- **Prof. dr. Vincent Timmerman, PhD**
  *Charcot-Marie-Tooth neuropathies: from genes to protein networks and disease mechanisms.*

## UCL

- **Dr. Fadel Tissir**
  *Celsr genes in brain development and function.*

## UGent

- **Prof. dr. Christophe Ampe**
  *ß-actin in neural crest cell migration and brain development.*

- **Prof. dr. Geert van Loo**
  *Study of the role of the NF-κB regulatory protein A20 in autoimmune central nervous system inflammation.*

## ULB

- **Dr. Eric Bellefroid**
  *Role of DMRT transcription factors in the development of the cerebral cortex.*

- **Prof. dr. Serge N. Schiffmann**
  *Roles of specific genes and neuronal populations in functions and disorders of basal ganglia.*

- **Dr. Pierre Vanderhaeghen, PhD**
  *From stem cells to cortical networks.*

## ULg

- **Prof. dr. Pierre Maquet**
  *Characterization of human sleep/Wake regulation using multimodal functional imaging in populations stratified on the polymorphism of PERIOD3 gene.*

- **Dr. Laurent Nguyen**
  *Unravelling the roles of lysine acetylation in neural development.*

## VUB

- **Prof. dr. Ilse Smolders**
  *Unveiling the role of the cystine/glutamate antiporter (system Xc-) in hippocampal functioning, mechanisms of epilepsy and its comorbidities: a new era for future drug treatment.*

# Progress reports of the university research groups, supported by the Queen Elisabeth Medical Fondation in collaboration with the following professors and doctors (2012)

Progress report of the research group of

# Prof. dr. C. Ampe

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# ß-actin in neural crest cell migration and brain development

## Background

The actin cytoskeleton is the driving force behind cell motility processes in health and diseases (Lambrechts *et al*., 2004). One main process during development that requires cell motility, is neural crest cell migration and subsequent neurogenesis (Kawauchi en Hoshino 2008). Indeed actin polymerization forms the propulsive force for cell migration, for neurite outgrowth and is an essential mediator in growth cone steering. In neuronal cells two actin isoforms are present: β- and γ- cytoplasmic actin (Tondeleir *et al*., 2009). It was long time accepted these isoforms were functionally redundant because they only differ in four amino acids. Recent studies employing mice show, however, these isoforms are not redundant but their differential roles are only slowly being unraveled. γ-actin knock-out (KO) mice are viable but show progressive hearing loss during adulthood, despite compensatory up-regulation of β-actin (Belyantseva *et al*., 2009) whereas β-actin KOs are embryonically lethal beyond E10.5 (Schmerling *et al*., 2005) despite up regulation of γ-actin and ectopic expression of α-smooth muscle actin at these stages (Tondeleir *et al*., 2012). Clearly the increased expression of other actin isoforms fails to rescue the lethal phenotype and does not compensate for lack of β-actin. Additionally expressing γ-actin from the β-actin locus does not rescue the lethal phenotype although occasionally embryos survive until E13.5-14.5 suggesting partial rescue (Lambrechts *et al*., unpublished). Various studies on conditional β-actin knock-outs (*Actb*$^{-/-}$) exist. Surprisingly, depletion of β-actin at late stages of motor neuron development or brain development yield only mild defects (Cheever *et al*., 2011; Cheever *et al.,* 2012). Viable central nervous system *Actb*$^{-/-}$ mice display restricted histological abnormalities in the hippocampus and cerebellum. These morphology defects correlated with hyperactivity and cognitive impairments (Cheever *et al.,* 2012). These restricted effects in vivo at late stages of brain development strongly contrast with the dramatic effects on NCC migration presented below.

β-actin is the form traditionally associated with cell migration but more and more a role for transcriptional regulation becomes evident (Visa and Percipalle, 2010). Primary embryonic fibroblasts (MEFs) isolated from the knock-out mouse have increased expression of γ- and α-smooth muscle actin and (re)differentiate to a myofibroblast-like phenotype. These cells display strongly impaired cell migration and increased adhesion. In addition we observed that the Rho – Rho-kinase (ROCK) pathway is over-activated and the MEFs have sustained transforming growth factor β (TGF-β) activity. Inhibiting ROCK or myosin contractility restores cell migration, strongly indicating that altered signaling is generating the impaired migration, rather than lack of actin polymerization capacity (Tondeleir *et al*. 2012). Collectively, our results point to an important role of actin in cell and organ differentiation via its essential nuclear function (Tondeleir *et al*. 2012, Tondeleir *et al.*, submitted A) and not via its traditional role as engine for cell motility. Also the result presented in the 2012 report on neural crest cells point in this direction (Tondelier *et al.*, submitted B).

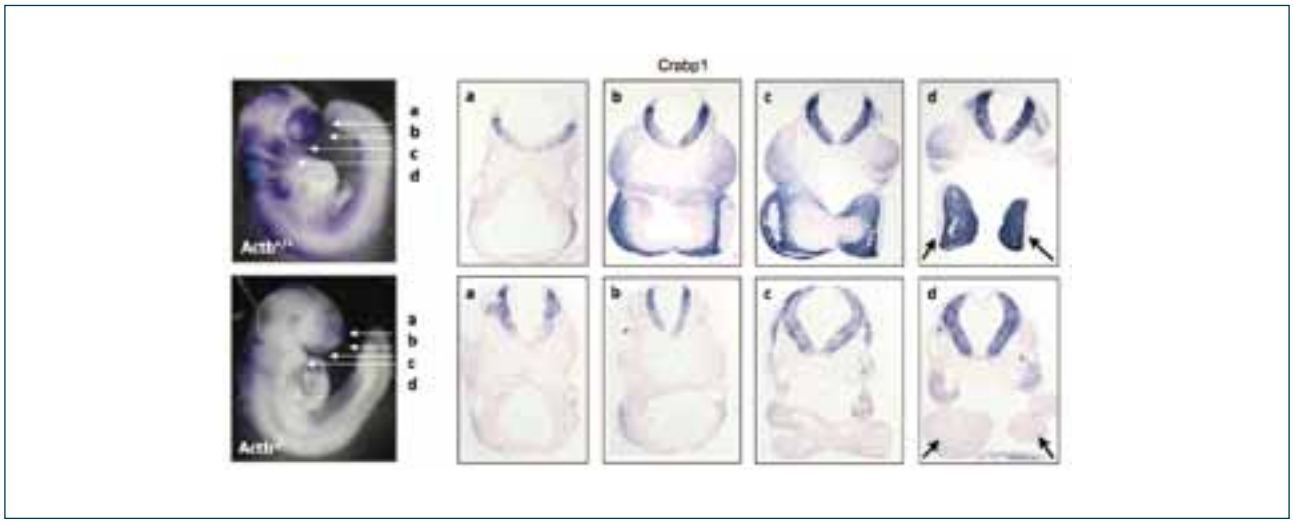
## We aim to better understand the role of β-actin in development in particular its role in neural crest cell migration and neurogenesis.

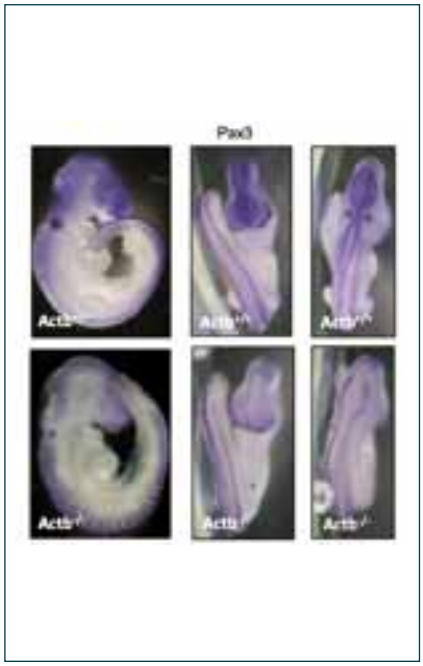
### A Defective neural crest cell migration in β-actin knock out embryos

We exploit the *Actb*$^{-/-}$ mouse model. Consistent with defective MEF migration, we observed in β-actin KO embryos phenotypes that can be attributed to impaired cell migration: e.g. aberrant vascularization (requiring endothelial cell migration) and, as documented in the 2011 report, an amazing lack of neurofilament staining at E9.5. We also showed that trunk neural crest cell (NCC) where specified but they displayed a migration defect. In addition *Actb*$^{-/-}$ NCC had a different morphology of wild type (*Actb*$^{+/+}$) NCC in neural explants. In contrast, to the *Actb*$^{-/-}$ MEFs (see introduction) we could only partially restore NCC migration using ROCK inhibition, suggesting that also different pathways were deregulated. This was a main topic of investigation in 2012 (see below).


We first corroborated the results obtained in 2011 in several ways. To probe the impaired formation of peripheral nervous system we tested for beta-III tubulin expression which is widely regarded as neuronal marker and one of the earliest markers to signal neuronal commitment in primitive neuroepithelium. Although this antibody gave a staining pattern in the *Actb*$^{-/-}$ embryos, aberrant coloration was seen compared to the *Actb*$^{+/+}$ control embryos. The trigeminal ganglion (V) of *Actb*$^{-/-}$ embryos is hypomorphic and formation of the glosso-pharyngeal (IX) and the vagus (X) nerves and the dorsal root ganglia (drg) are largely impaired.

Next to Sox10 expression (report 2011) we monitored Crap1 expression in different sections (white arrows) at stage E9,5 which confirmed the migration defects of the trunk neural crest cells in the *Actb*$^{-/-}$ embryos. These embryos show no or few neural crest cells in regions distant from the neural tube, indicating a severe migration problem (black arrows). Closer to the neural tube some migrated NCC cells can be however occasionally observed (lower panel C)






In addition whole mount in situ hybridization of Pax3 was done. Pax3 is expressed during early cranial neurogenesis and in the dermomyotome component of the somites (Bober et al., 1994; Conway et al., 1997). While the expression of pax3 remained identical between Actb$^{+/+}$ embryos and *Actb*$^{-/-}$ embryos in the trunk region, the cranial region of the *Actb*$^{+/+}$ embryos displayed substantial less pax3 coloring, again indicating a neural crest migration problem.

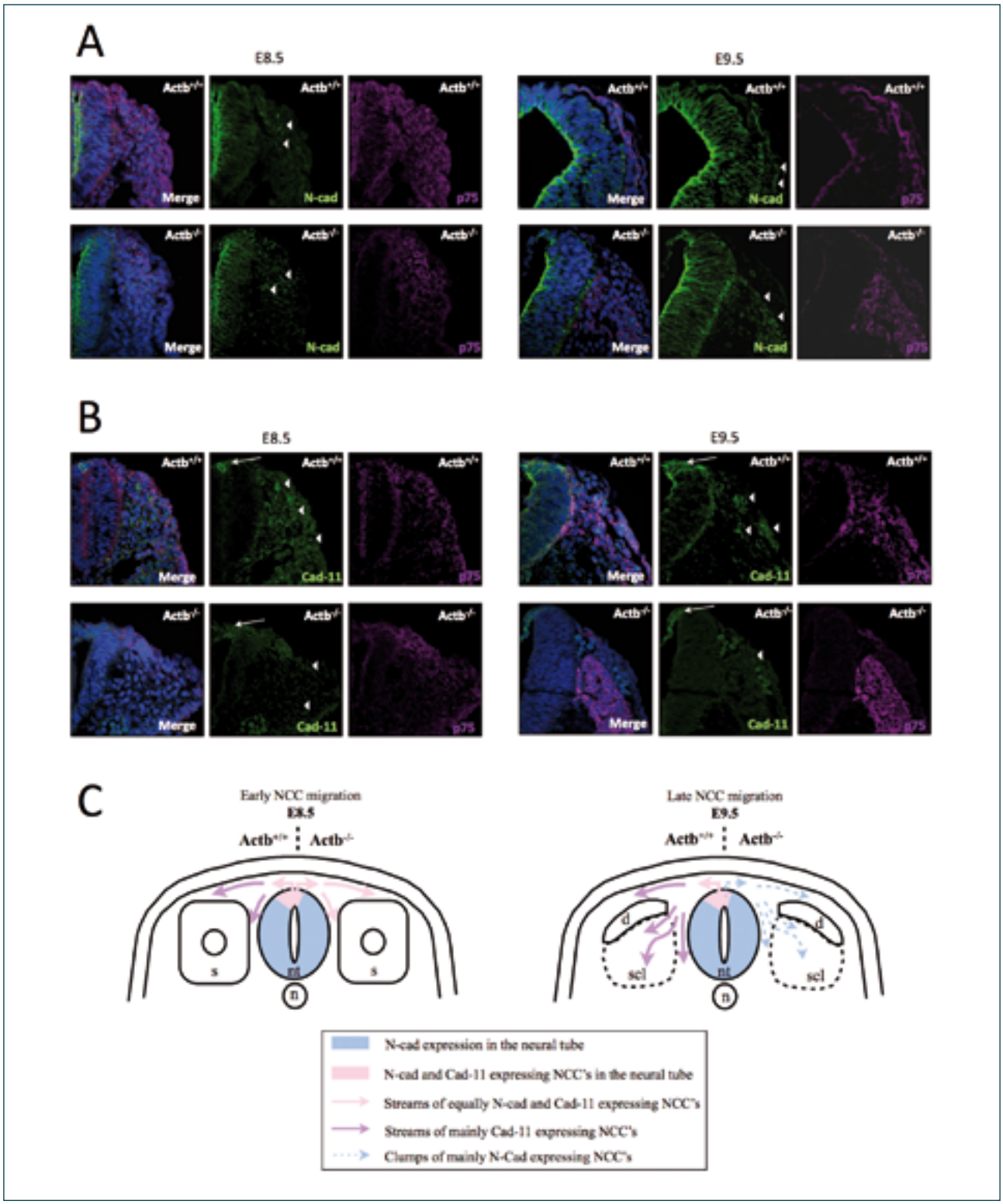
Given the observed phenotype of the NCC in explants (lack of single cell migration and behaviour as a sheet, see figures in report 2011) and given the importance of cadherins in cell-cell interactions and the connection with actin cytoskeleton we paid attention to cadherin switching which occurs during NCC epithelial mesenchymal transition (EMT) prior to delamination in other animal models (Acloque *et al*., 2009, cadherin switching during neural crest ontogony is poorly documented for mouse in literature).

## Aberrant cadherin expression in neural crest cells of Actb$^{-/-}$ embryos in vivo

The *Actb*$^{-/-}$ MEFs display altered signaling in part due to a changed genetic program, including differential expression of adhesion molecules (Tondeleir *et al*., 2012). Although the nature and the extent of altered signaling in *Actb*$^{-/-}$ neural crest cells is unknown we probed expression of cadherins to investigate if altered cadherin expression played a role in this phenotype. It has been well established, in other model organisms, that cadherins play a significant role in cell sorting and the regulation of tissue formation and in neural crest cell migration (Theveneau and Mayor, 2012, Wheelock and Johnson, 2003).

We devoted time to optimize protocols for probing using immune-fluorescence for different cadherins at stage E10,5 in WT mice. Although we could not establish good conditions yet for cadherin-6 and 7 we did manage probing E-, N-cadherin and cadherin 11 (Noelanders Master thesis 2012). Sectioning the embryos at stages E8.5 and E9.5 enabled us to probe expression of these cadherins at these two stages of neural crest ontogeny. We used p75 as a marker to track the neural crest in combination with the different cadherin antibodies. At E8.5, we could not detect major differences in the expression pattern of N-cadherin and E-cadherin between *Actb*$^{+/+}$ and *Actb*$^{-/-}$ embryos. Unexpectedly E-cadherin expression at this stage was not detectable in the neural tube or in migrating neural crest cells close to the neural tube in both *Actb*$^{+/+}$ and *Actb*$^{-/-}$ embryos. At E9,5 E-cadherin showed no expression in neither of the embryos. At both stages N-cadherin expression was present in the whole neural tube and remained present in the migrating neural crest cells, albeit to a lower extent relative to the neural tube) (panel A). In E8.5 and E9.5 *Actb*$^{+/+}$ embryos cadherin-11 is present in neural crest cells in the dorsal neural tube en remains strongly expressed in the migratory neural crest (panel B). However, cadherin-11 was present to a much lower amount in *Actb*$^{-/-}$ embryos versus the *Actb*$^{+/+}$ embryos, both in the dorsal neural tube as in migrating neural crest cells. N-cadherin expression levels are higher in migratory p75 positive cells of *Actb*$^{-/-}$ embryos versus *Actb*$^{+/+}$ embryos (panel A).

With respect to migration, at E8.5, one can still appreciate the streams of invading neural crest cells as visualized by the p75 antibody. At this point there is no major difference in the patterns between the *Actb*$^{+/+}$ and *Actb*$^{-/-}$ embryos. However, in the *Actb*$^{-/-}$ embryos at E9.5 most migrating neural crest cells were organized as clumps rather than streams, indicating that, even close to the neural tube, well organized migration of neural crest cells is disturbed after E8.5. Panel C shows a schematic view of the common and differential cadherin expression patterns in *Actb*$^{+/+}$ and *Actb*$^{-/-}$ embryos at both developmental stages.



In summary we show an essential role for β-actin in neural crest cell ontogeny. The observed NCC migration defect is likely due to both altered post-transcriptional signaling (at least in the Rho pathway that is connected to cadherin signaling) and an improper EMT in the NCC cells resulting in aberrant cadherin expression. These data are consistent with an upstream regulatory role of β-actin suggesting again that its nuclear function, and not its cytoskeletal function, is the more important one.
This work is part of the PhD doctoral thesis of Davina Tondeleir, submitted January 7th, defense foreseen end of March.

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Progress report of the research group of

# Prof. dr. C. Bagni

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# mRNA metabolism at synapses and spine remodeling: insights into Fragile X, Autism and Schizophrenia

## A STATE OF THE ART AND SUMMARY OF THE RESEARCH PROGRAM

Memory formation and cognitive processes rely on activity-dependent synaptic plasticity. Synaptic inputs dictate the time, place and amount of protein synthesis necessary for the single synapses. Dysregulation of these mechanisms leads to spine dysmorphogenesis (Fiala et al., 2002) and to a variety of pathological conditions including the most common form of inherited mental retardation due to the absence or mutation of a single protein, FMRP (Bagni et al., 2012). FMRP is involved in multiple steps of neuronal messenger RNA metabolism such as transport, stability and local translation. We have shown that FMRP, together with its cytoplasmic interactor CYFIP1, controls, in an activity dependent manner, the synthesis of key proteins at synapses, which are impaired in a mouse model for FXS.
The protein CYFIP1 has a critical role in human brain function/s. Deletions and/or duplications of CYFIP1 have been associated to Autism, Schizophrenia and Epilepsy (Bittel et al., 2006; Chai et al., 2003; Doornbos et al., 2009; Tam et al., 2010; van der Zwaag et al., 2010; von der Lippe et al., 2010). Using a mouse model (Cyfip1 KO) and other model systems (Bozdagi et al., 2012; Schenck et al., 2003; Star et al., 2002) it has been shown that CYFIP1 is involved in the formation of synaptic processes. Therefore unbalances in CYFIP1 interactive network/s might result in neuronal dysfunctions as dendritic spines dysgenesis: a common cellular phenotype observed in patients with Fragile X Syndrome and other mental disorders (Fiala et al., 2002).
In the proposed project we plan to: 1) Identify and characterize the CYFIP1 complexes in different neuronal compartments, 2) Investigate whether the Rho GTPase Rac1, a CYFIP1 interactor, regulates CYFIP1-dependent protein synthesis, 3) Study the physiological regulation of FMRP and CYFIP1 upon synaptic activation.
**This project will shed light into mental retardation, Autism and possibly schizophrenia.**

## B RESULTS

To understand the role of CYFIP1 in all these complex diseases, we have identified CYFIP1 synaptic complexes and characterized its neuronal functions through the following aims:

***Aim 1***:
Identification and characterization of the CYFIP1 complexes in different neuronal compartments, i.e. cortex, hippocampus and isolated synapses.

***Achievement 1:***
To isolate the CYFIP1 complexes, we have immunoprecipitated CYFIP1 using a specific antibody (Napoli et al., 2008). The co-interacting proteins were then analyzed by liquid chromatography/mass spectrometry analysis (LC-MS/MS), in collaboration with the group of Prof. August B. Smit, Dept. of Molecular and Cellular Neurobiology, Center for Neurogenomics and Cognitive Research, VU University Amsterdam, The Netherlands. We performed electron microscopic (EM), and immunohistochemistry (IHC) studies that indicate that CYFIP1 and FMRP are enriched at synapses. We next focused on this highly specialized subcellular compartment which, as previously mentioned, is largely affected in several neurological syndromes and in mental retardation (Fiala et al., 2002). At synapses, we identified 35 novel

CYFIP1 interacting proteins, in addition to other 4 already identified in other studies, including RNA-binding proteins implicated in mRNA metabolism and actin cytoskeleton regulators. Intriguingly, the composition of the complexes changes according to the their neuronal subcellular localization. Since CYFIP1 has been related to intellectual disability, autism and schizophrenia, we investigated whether the genes encoding the CYFIP1 partners are involved in neuropsychiatric disorder by disease annotation based on published literature. Importantly, we have found that 2/3 of the novel CYFIP1 interactors show a significant association with intellectual disability, autism and schizophrenia.
We have now further deepened the understanding of CYFIP1 involvement in those diseases by gene-based analysis for association with autism and schizophrenia; for this purpose, interrogated meta-analysis data performed by Genome-Wide Association Study (GWAS) Consortia in collaboration with Dr. Danielle Postuma at the VU University Amsterdam, The Netherlands. Of note, we discovered that, eight novel CYFIP1 interactors are key proteins involved in the functioning of the translational machinery, RNA metabolism and actin remodeling. Next we have obtained human brain specimens (*post mortem* brain tissues from patients with schizophrenia, depression and bipolar disorder and unaffected controls) from the Stanley Consortium (https://www.stanleygenomics.org/collect.html) and initiated the study of the expression profile of several CYFIP1 interactors including FMRP. Our preliminary data show that FMRP (Fragile X Mental Retardation Protein, a key interactor of CYFIP1 is dysregulated in Schizophrenia and Bipolar disorder patients (De Rubeis and Bagni, unpublished). Finally we observed a different CYFIP1 level in patients with ASD (Borreca and Bagni, unpublished). **These initial findings will be now extensively validated in human postmortem brains (different brain areas and diseases) and may possibly lead to the identification of new genes/markers for such complex disorders.**

***Aim 2:***
CYFIP1-mediated regulation of actin cytoskeleton remodeling and local protein synthesis.

**Achievement 2:**
The CYFIP1 interactome revealed the existence of two complexes: 1) CYFIP1-FMRP-eIF4E, implicated in translational control and 2) the WAVE Regulatory Complex (WRC), involved in remodeling of the actin cytoskeleton. Therefore, we hypothesized that the activation of small GTPases upstream of the WRC, might release CYFIP1 from eIF4E and relocating it on the WRC. We were able to prove that those events occur as response to synaptic activity. We have now directly studied the dynamics of CYFIP1-eIF4E interaction in synaptoneurosomes stimulated with BDNF in presence or absence of GTPases inhibitors and showed that the effects of those inhibitors on activity-induced local protein synthesis are dependent on CYFIP1 and/or FMRP. **We are now in the process of making CYFIP1 mutants (for the domain regulating actin remodeling and the domain regulating local protein synthesis) to further the functions of CYFIP1 at synapses. The precise regulation of these two complexes may help the design of possible drugs to ameliorate some of these diseases.**

***Aim 3:***
Regulation of FMRP and CYFIP1 upon neuronal activity.

**Achievement 3:**
Translational initiation, in particular sequestration of eIF4E by 4E-binding proteins, is tightly controlled by the mTORC1 pathway. mTORC1 can be activated by a variety of receptors, including the metabotropic glutamate receptors (mGluRs). To investigate if this signaling cascade affects not only general 4E-BPs, but also specific ones such as CYFIP1, we stimulated acute hippocampal slices with a group I mGluRs agonist and simultaneously block mTORC1 with rapamycin. We found that CYFIP1 dissociates from

eIF4E upon DHPG stimulation, but Rapamycin prevented this effect, thus indicating that mTORC1 is required to release CYFIP1 upon mGluRs. **We are now currently studying the phosphorylation state of CYFIP1 by phosphoproteomics, in collaboration with Prof. Kris Gevaert (University of Gent). We have immunoprecipitated CYFIP1 from unstimulated and DHPG-treated synaptoneurosomes and analyzed the presence of phosphopeptides by MS. These studies are currently on progress and will allow us to understand how neuronal activity modulates the two complexes via posttranslational modifications.**

**Achievement 4.**
Furthermore, since translational control and actin cytoskeleton rearrangements are very important for dendritic spine morphology, we have investigated the effects of CYFIP1 depletion on spine morphology, by knocking down *Cyfip1* with specific shRNAs in primary cortical neurons and studying spine density and morphology. **Our data show that CYFIP1 is indeed a key player in neuronal spine formation and strongly suggest that its involvement in Autism, Schizophrenia and Epilepsy is indeed caused by a dysregulated neuronal connectivity.**

## C NETWORKING AND COLLABORATIONS

The project results from the integration of complementary expertise in our Institute and at other institutions abroad. First, the mass spectrometry analysis was conducted in collaboration with a group with a long lasting experience in mass spectrometry techniques, namely the group of Prof. August B. Smit and Dr. Ka Wan Li, at the VU University in Amsterdam, The Netherlands (Klemmer et al., 2009; Li et al., 2005; Li et al., 2006; Li et al., 2007; Li and Smit, 2008). Furthermore, in the past year we have also collaborated with clinical geneticists for the GWAS analysis (Dr. Danielle Posthuma, Department of Functional Genomics and Department of Medical Genomics, VU Amsterdam, The Netherlands) and with European consortia and networks. Finally, we are proficiently collaborating with the microscope imaging facility at the Center of Human Genetics, KU Leuven (Light Microscopy & Imaging Network, LiMoNe) for spine visualization and analysis. We have finally started a new collaboration with Prof. Kris Gevaert to further study the CYFIP1 regulation via the identification of post-translational modifications.

## D RELEVANCE

The work performed so far greatly contributed to the current knowledge of CYFIP1 distribution and function/s in brain and provides novel mechanisms that explain the molecular aspects of several intellectual disabilities.

- We have identified as partner of CYFIP1, new genes/proteins involved in Autism, Schizophrenia and other psychosis in humans.
- We have increased our understanding on the function of synaptic compartment in the neuronal cells unraveling the different, interconnected CYFIP1 cellular functions.
- We have shed light on the regulatory mechanisms tuning CYFIP1 complexes with neuronal stimulation.

**The isolation and functional characterization of the CYFIP1 interactome, a protein implicated in several pathologies, helps to understand the interconnection and co-morbidity between different neuropsychiatric disorders. We hope that our studies will very soon set up the ground for drug screening to ameliorate those disorders.**

## E PUBLICATIONS supported by the F.M.R.E

- **Claudia Bagni,** Flora Tassone, Giovanni Neri and Randi Hagerman (2012). "Fragile X Syndrome Year 2012: Causes, Diagnosis, Mechanisms and Therapeutics". ***J. Clin. Invest.,***122: 4314-2.
- Silvia De Rubeis, Ka Wan Li, Andrea Buzzi, Emanuela Pasciuto, Bing Yang, Daniele Di Marino, Esperanza Fernandez, Linnaea E. Ostroff, Fried Zwartkruis, Eric Klann, Noburo H. Komiyama, Seth Grant, Tilmann Achsel, Danielle Posthuma, August B. Smit and **Claudia Bagni**. CYFIP1 co-ordinates mRNA translation and cytoskeleton remodeling to ensure proper dendritic spine formation. (**Neuron**, pending revision).

## F TEAM PUBLICATIONS (last five years).

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Progress report of the research group of

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# Role of DMRT transcription factors in the development of the cerebral cortex

The mammalian cerebral cortex is divided into distinct cytoarchitectonic areas that serve specialized functions. The prevaling model of how distinct cortical areas arise from an initially homogenous and multipotent neuroepithelium is that signaling centres (the anterior commissure, cortical hem,…) secrete morphogens that provide progenitors with positional information that « patterns » the neuroepithelium. These signals establish the graded expression of transcription factors in progenitors which is crucial for the early regionalisation and subsequent arealization of the cortex. To date, five transcription factors (Emx2, Pax6, COUP-TF1, Sp8 and Lhx2) have been shown to be expressed in progenitors and have a role in arealization (Hébert and Fishell, 2008; O'Leary DD, Sahara S, 2008; Borello U, Pierani A 2010). How these factors function together to control arealization is one major challenge in the field of cortical development. Whether additional genes that are differentially expressed within the cortex play also a role in arealization is currently the subject of much efforts.

The *Dmrt d*oublesex and *m*ab3-*r*elated-*t*ranscription factor (Dmrt) genes encode a large family of transcription factors with an unusual zinc finger DNA binding domain that are well known for their important role in gonad development and sexual differentiation in arthropodes, nematodes and vertebrates. In vertebrates, several members of this family are also strongly expressed in non-gonadal tissues and play important functions during embryogenesis. We recently found that the *Dmrt5* gene is expressed in a graded manner within the cortical neuroepithelium and generated a knock-out mice to analyse its function. Our results showed that Dmrt5 plays a key role in cortical development and suggest that it does so through the promotion of the production of Wnt and Bmp signaling molecules in the cortical hem and the modulation of the graded expression of the known downstream transcription factors specifying cortical identity. This work constitutes the first demonstration of the important role of a member of this gene family in cortical development. A manuscript by Saulnier et al. entitled "**The doublesex homolog Dmrt5 is required for the development of the caudomedial cerebral cortex in mammals**. » describing those results has been accepted for publication in the revue ***Cerebral cortex*.**

During this year, we also focused on the characterization of the Dmrt5 ortholog we identified in the frog *Xenopus laevis* that, as in the mouse, is strongly expressed in the developing telencephalon and olfactory system. The inductive events that lead to olfactory placode development remain unclear (Schlosser, 2006; Streit, 2008; Schlosser, 2010; Park et al., 2010). We therefore analysed the regulatory inputs that control *Dmrt5* expression in the ectoderm and the consequences of its knockdown and overexpression on neurogenesis within the developing olfactory epithelium. Our results showed that the *Dmrt5* gene is a novel important player in the developing olfactory system, induced by the events of neural induction and the integration of the inputs of the homeobox transcription factor Otx2 and of Notch signaling, and provide evidence for *Dmrt5* and *Dmrt4* redundant functions upstream of proneural factors. This work constitutes the first demonstration of the important role of Dmrt5 in neurogenesis during olfactory placode development. A manuscript by Parlier et al. entitled "**The Xenopus doublesex-related gene Dmrt5 is required for olfactory placode neurogenesis.** » describing those results has been accepted for publication to the journal ***Dev. Biol.***

A review on the structure and evolution of *Dmrt* genes and of their embryonic expression pattern across vertebrate species, summarizing recent findings on their function and highlighting the important role of a subgroup of them including *Dmrt3*, *Dmrt4* and *Dmrt5* in neurogenesis and patterning of the developing nervous system has been written. This revue, entitled "**Expanding roles for the evolutionarily conserved *Dmrt* sex transcriptional regulators during vertebrate embryogenesis"** is under revision for ***Cellular and Molecular Life Sciences (CMLS).***

Besides our work on cortical development, we have also been engaged in the study of the molecular mechanisms that control spinal cord neurogenesis. Our work fouses on two members of the Prdm transcription factor family. This family has recently spawned considerable interest as it has been implicated in fundamental aspects of cellular differentiation and exhibits expanding ties to human diseases (Fog et al., 2011 ; Hohenauer et al., 2012). In an *in situ* hybridization screen, we recently identified in the frog embryo several uncharacterized members of this family, including Prdm12 and Prdm13. These genes are expressed in restricted progenitors of the developing hindbrain and spinal cord, which suggests a function for them in neuronal specification. As for *Prdm12*, we have obtained evidence indicating that it plays a crucial role in spinal cord V1 interneuron (IN) specification. The precise roles and mechanisms of action of Prdm12 is currently under investigation. As for *Prdm13*, our recent data indicate that it constitutes a novel downstream target of Ptf1a, a basic helix-loop-helix (bHLH) proneural factor that determines GABAergic neuronal fate in the dorsal spinal cord. Its importance downstream of Ptf1a is currently under study.

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- Eric J. Bellefroid, Lucas Leclère, Amandine Saulnier, Marc Keruzore, Maria Sirakov, Michel Vervoort, Sarah De Clercq. Expanding roles for the evolutionarily conserved *Dmrt* sex transcriptional regulators during vertebrate embryogenesis. In revision for ***Cell Mol. Life Sci.***
- Hanotel J., Thélie A., Bellefroid E.J. The zinc finger transcription factor Prdm13 is a direct Ptf1 target in the developing Xenopus spinal cord. In preparation.
- Thélie A., Hanotel J., Francius C., Cerda G., Lewis K., Clotman F., Bellefroid E.J. The zinc finger transcription factor Prdm12 plays an essential role in spinal cord V1 interneuron specification. In preparation.

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# Molecular Genetics and Functional Genomics of Frontotemporal Lobar Degeneration

## Scientific report

### Specific aims

In this research project we aimed to expand our understanding of the biochemical pathways that contribute to the etiology of frontotemporal lobar degeneration (FTLD) using state-of-the-art molecular genetics and genomics strategies.

The specific objectives specified in the research project were to
- extend our FTLD patient and matched control samples
- identify genes modifying onset age in FTLD
- identify novel causal genes for FTLD
- identify novel susceptibility genes for FTLD

In 2012, major research results were obtained for objectives 1, 3 and 4. We followed up on the major finding of the FTLD-ALS gene *C9orf72* at chromosome 9p, that we identified in 2011. Further, knock-out mice for granulin (*GRN*), the FTLD gene that we identified in 2006, were generated and characterized. The progress we made towards the research objectives are reported. In addition, we reported on the structure and content of the Alzheimer disease and frontotemporal dementia (AD&FTLD) and Parkinson disease (PD) Mutation Databases that we maintain for many years. They are established resources for clinical geneticists, neurologists, and researchers in need of comprehensive, referenced genetic, epidemiologic, clinical, neuropathological, and/or cell biological information of specific gene mutations in these diseases. In addition, the aggregate analysis of all information available in the databases provides unique opportunities to extract mutation characteristics and genotype-phenotype correlations, which would be otherwise unnoticed and unexplored (Cruts et al., 2012, http://www.molgen.ua.ac.be/FTDMutations).

### Causal genes for FTLD

There is increasing evidence that FTLD and amyotrophic lateral sclerosis (ALS) represent a continuum of neurodegenerative diseases. FTLD is complicated by ALS in a significant proportion of patients, and neuropsychological studies have demonstrated frontotemporal dysfunction in up to 50% of ALS patients. More recently, advances in neuropathology and molecular genetics have started to disclose the biological basis for the observed clinical concurrence. TDP-43 and FUS have been discovered as key pathological proteins in both FTLD and ALS. The most recent discovery of a pathological hexanucleotide repeat expansion in the gene C9orf72 as a frequent cause of both FTLD and ALS has eventually confirmed the association of these two at first sight distinct neurodegenerative diseases (Van Langenhove et al., 2012b). We published a major paper describing the identification of this GGGGCC ($G_4C_2$) hexanucleotide repeat expansion in the regulatory region of *C9orf72*, that explained genetic linkage and association at chromosome 9p13-21 locus in FTLD, ALS and FTLD-ALS (Gijselinck et al., 2012). In our patient collection, 86%, 47% and 16% of patients with inherited FTLD-ALS, ALS, and FTLD respectively were explained by this genetic defect. The paper was published online in 2011 and

was reported in detail in the 2011 report. Here we report on an international follow-up study and on the exploration of the effect of the repeat expansion on gene expression.
In 2006, we identified *GRN* null mutations as a major gene for FTLD explaining 26 % of familial FTLD in Belgium (Cruts et al., 2006). Now, we published a paper describing the generation and detailed genetic, clinical, behavioral and immunohistochemical characterization of *GRN* knock-out mize.

### *C9orf72*

We assessed the geographical distribution of *C9orf72* $G_4C_2$ repeat expansions in a collection of 1,205 European FTLD patients, ascertained by the European Early-Onset Dementia (EOD) consortium. Next, we performed a meta-analysis of these data and that of other European studies, together 2,668 patients from 15 Western European countries. The frequency of *C9orf72* repeat expansions in Western Europe was 9.98%, with 18.52% in familial, and 6.26% in sporadic FTLD patients. Outliers were Finland and Sweden with overall frequencies of respectively 29.33% and 20.73%, but also Spain with 25.49%. In contrast, prevalence in Germany was limited to 4.82% (van der Zee and Gijselinck et al., 2012).

Intermediate repeat sizes (7 to 24 repeat units) are strongly correlated with a haplotype conferring significant risk especially for developing ALS and FTLD-TDP (van Es et al., 2009; Laaksovirta et al., 2010; Shatunov et al., 2010; Van Deerlin et al., 2010). We studied the role of intermediate repeat sizes on *C9orf72* gene expression. *In vitro* reporter gene expression studies demonstrated significantly decreased transcriptional activity of *C9orf72* with increasing number of repeat units (Figure 1), indicating that intermediate repeats might act as predisposing alleles and in favor of the loss-of-function disease mechanism. Further, we observed a significantly increased frequency of short indels in the GC-rich low complexity sequence (LCS) adjacent to the $G_4C_2$ repeat in *C9orf72* expansion carriers ($p < 0.001$) with the most common indel creating one long contiguous imperfect $G_4C_2$ repeat which is likely more prone to replication slippage and pathological expansion (van der Zee and Gijselinck et al., 2012).

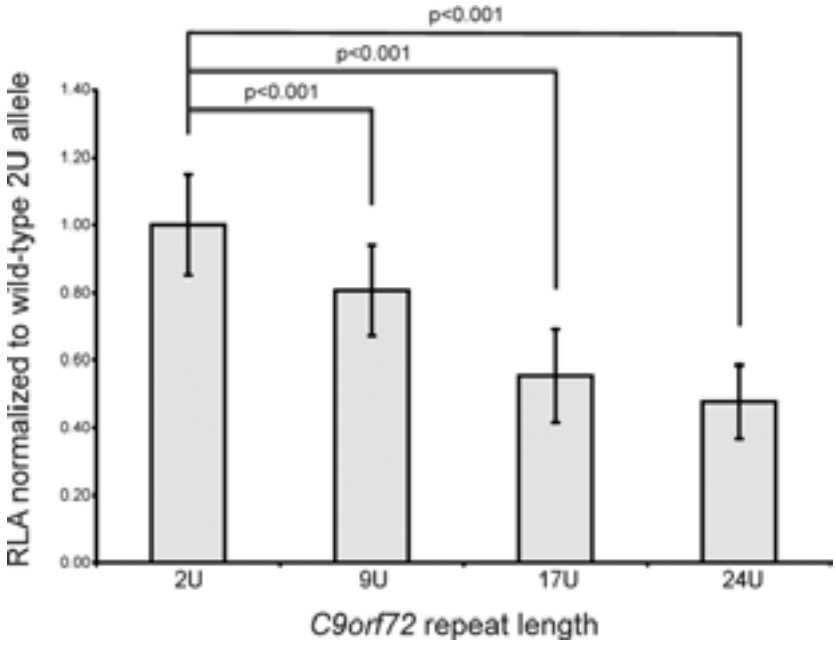


**Figure 1. Transcriptional activity of C9orf72 promoter with alleles of different repeat length.** Bars represent relative Gaussia/Cypridina luciferase activities (RLA) for the different C9orf72 constructs compared to the wild type allele of 2 units, for an increasing amount of repeat units. Values represent the mean (±SDEV) of 36 independent measurements relative to the 2 units wild type allele. The significance of differences in expression was calculated using the Mann-Whitney U test. P-values are presented above the bars (van der Zee and Gijselinck et al., 2012).

### Granulin (*GRN*)

Loss-of-function mutations in granulin (*GRN*) are associated with FTLD with intraneuronal ubiquitinated protein accumulations composed primarily of hyperphosphorylated TDP-43 (FTLD-TDP). The mechanism by which *GRN* deficiency causes TDP-43 pathology or neurodegeneration remains elusive. To explore the role of *GRN in vivo*, we established *Grn* knockout mice using a targeted genomic recombination approach and Cre-LoxP technology. Constitutive *Grn* homozygous knockout ($Grn^{-/-}$) mice were born in an expected Mendelian pattern of inheritance and showed no phenotypic alterations compared to heterozygous ($Grn^{+/-}$) or wild-type (Wt) littermates until 10 months of age. From then, $Grn^{-/-}$ mice showed reduced survival accompanied by significantly increased gliosis and ubiquitin-positive accumulations in the cortex, hippocampus, and subcortical regions. Although phosphorylated TDP-43 could not be detected in the ubiquitinated inclusions, elevated levels of hyperphosphorylated full-length TDP-43 were recovered from detergent-insoluble brain fractions of $Grn^{-/-}$ mice. Phosphorylated TDP-43 increased with age and was primarily extracted from the nuclear fraction. $Grn^{-/-}$ mice also showed degenerative liver changes and cathepsin D-positive foamy histiocytes within sinusoids, suggesting widespread defects in lysosomal turnover. An increase in insulin-like growth factor 1 (IGF1) was observed in $Grn^{-/-}$ brains, and increased IGF1 signaling has been associated with decreased longevity. Our data suggest that progranulin deficiency in mice leads to reduced survival in adulthood and increased cellular ageing accompanied by hyperphosphorylation of TDP-43, and recapitulates key aspects of FTLD-TDP neuropathology (Wils et al., 2012).

## Susceptibility genes for FTLD

### Ataxin 2 (*ATXN2*)

Considerable clinical and pathological overlap exists FTLD and ALS, which implies that these 2 neurodegenerative conditions share common pathogenic mechanisms. Recently, intermediate-length (27-33) polyglutamine (polyQ) expansions in *ATXN2* have been associated with increased risk for ALS, while expansions of > 34 repeats are known to cause spinocerebellar ataxia type 2 (SCA2). We identified in 72 ALS patients one patient with a $Q_{33}$ expansion that was absent in 810 control individuals. This allele was also found in one patient with concomitant ALS-SCA2. In contrast, in a Flanders-Belgian series of 270 FTLD and 22 FTLD-ALS patients, we found no association with intermediate-length polyQ expansions nor did we observe patient-specific long expansions in agreement with the recent observation in a screening of a substantial sized cohort of patients with diverse neurodegenerative brain diseases. Our results provide further support to the notion that *ATXN2* associated polyQ amplification is specific to the ALS-end of the FTLD-ALS disease spectrum (Van Langenhove et al., 2012a).

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# Publications Acknowledging G.S.K.E. Funding

## Articles in international journals

- **Gijselinck,I.**, Van Langenhove,T., **van der Zee,J.**, Sleegers,K., Philtjens,S., Kleinberger,G., Janssens,J., Bettens,K., Van Cauwenberghe,C., Pereson,S., Engelborghs,S., Sieben,A., De Jonghe,P., Vandenberghe,R., Santens,P., De Bleecker,J., Maes,G., Bäumer,V., Dillen,L., Joris,G., Cuijt,I., Corsmit,E., Elinck,E., Van Dongen,J., Vermeulen,S., Van den Broeck,M., Vaerenberg,C., Mattheijssens,M., Peeters,K., Robberecht,W., Cras,P., Martin,J-J., De Deyn,P., **Cruts,M.**, Van Broeckhoven,C.: A C9orf72 promoter repeat expansion in a Flanders-Belgian cohort with disorders of the frontotemporal lobar degeneration-amyotrophic lateral sclerosis spectrum: a gene identification study. Lancet Neurology 11(1): 54-65 (2012) Epub: 07-Dec-2011 (PMID: 22154785) (I.F.: 21.659)
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- Sieben,A., Van Langenhove,T., Engelborghs,S., Martin,J-J., Boon,P., Cras,P., De Deyn,P., Santens,P., Van Broeckhoven,C., **Cruts,M.**: The genetics and neuropathology of frontotemporal lobar degeneration Acta Neuropathologica 124(3): 353-372 (2012) Epub: 14-Aug-2012 (PMID: 22890575) (I.F.: 9.32)
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- **Cruts,M.**, Theuns,J., Van Broeckhoven,C.: Locus-specific mutation databases for neurodegenerative brain diseases. Human Mutation 33(9): 1340-4 (2012) Epub: 02-Jul-2012 (PMID: 22581678) (I.F.: 5.686)
- **van der Zee,J.**, **Gijselinck,I.**, Dillen,L., Van Langenhove,T., Theuns,J., Engelborghs,S., Philtjens,S., Vandenbulcke,M., Sleegers,K., Sieben,A., Bäumer,V., Maes,G., Corsmit,E., Borroni,B., Padovani,A., Archetti,S., Perneczky,R., Diehl-Schmid,J., De Mendonca,A., Miltenberger-Miltenyi,G., Pereira,S., Pimentel,J., Nacmias,B., Bagnoli,S., Sorbi,S., Graff,C., Chiang,H.-H., Westerlund,M., Sanchez-Valle,R., Llado,A., Gelpi,E., Santana,I., Rosario Almeida,M., Santiago,B., Frisoni,G., Zanetti,O., Bonvicini,C., Synofzik,M., Maetzler,W., Müller vom Hagen,J., Schöls,L., Heneka,M.T., Jessen,F., Matej,R., Parobkova,E., Kovacs,G.G., Ströbel,T., Sarafov,S., Tournev,I., Jordanova,A., Danek,A., Arzberger,T., Fabrizi,G.-M., Testi,S., Salmon,E., Santens,P., Martin,J-J., Cras,P., Vandenberghe,R., De Deyn,P.P., **Cruts,M.**, Van Broeckhoven,C., on behalf of the European Early-Onset Dementia (EOD) Consortium,: A Pan-European study of the C9orf72 repeat associated with FTLD: geographic prevalence, genomic instability and intermediate repeats. Human Mutation (2012) Epub: 30-Oct-2012 (PMID: 23111906) (I.F.: 5.686)
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## Articles in books

- **Gijselinck,I.**, Sleegers,K., Van Broeckhoven,C., **Cruts,M.**: A major genetic factor at chromosome 9p inplicated in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). In: Amyotrophic Lateral Sclerosis Edited by Martin H. Maurer (InTech): 537-554 (2012)

## Abstracts in abstract books of international meetings

- **Gijselinck,I., van der Zee,J.,** Dillen,L., Van Langenhove,T., Philtjens,S., Engelborghs,S., Vandenbulcke,M., Bäumer,V., Maes,G., Santens,P., Cras,P., Robberecht,W., De Jonghe,P., Vandenberghe,R., De Deyn,P., Van Broeckhoven,C., **Cruts,M**.: Genomic Characterization of the C9orf72 Repeat Region Associated with FTLD and ALS. International Conference on Frontotemporal dementia, Manchester, UK, September 5-7 (2012) Dementia and Geriatric Cognitive Disorders 34(S1): 69
- **Gijselinck,I.**, Van Langenhove,T., **van der Zee,J.**, Philtjens,S., Engelborghs,S., De Jonghe,P., Vandenberghe,R., Santens,P., De Bleecker,J., Bäumer,V., Maes,G., Dillen,L., Cras,P., Robberecht,W., De Deyn,P., Van Broeckhoven,C., **Cruts,M.**: Functional effects of the MAPT haplotypes. Alzheimer's & Dementia - Supplement AAIC : (2012) (I.F.: 6.373)
- Philtjens,S., **Gijselinck,I.,** Van Langenhove,T., **van der Zee,J**., Engelborgs,S., Vandenberghe,R., Vandenbulcke,M., Santens,P., De Deyn,P., Van Broeckhoven,C**., Cruts,M**.: Identification of novel causal genes for frontotemporal lobar degeneration using whole genome sequencing. International Conference on Frontotemporal dementia, Manchester, UK, September 5-7 (2012) Dementia and Geriatric Cognitive Disorders 34(S1): 9
- Philtjens,S., **Gijselinck,I.**, Van Langenhove,T., **van der Zee,J.**, Engelborghs,S., Vandenberghe,R., Santens,P., De Deyn,P., Van Broeckhoven,C., **Cruts,M.**: In search of genetic defects in unrelated frontotemporal lobar degeneration patients using

whole genome sequencing. European Journal of Human Genetics - Supplement ESHG P12.163: (2012) (I.F.: 4.4)
- **van der Zee,J., Gijselinck,I.,** Dillen,L., Van Langenhove,T., Sieben,A., Martin,J.J., **Cruts,M**., Van Broeckhoven,C., European EOD Consortium: A Pan-European study of the C9orf72 expansion associated with FTLD and ALS. International Conference on Frontotemporal dementia, Manchester, UK, September 5-7 (2012) Dementia and Geriatric Cognitive Disorders 34(S1): 78
- **van der Zee,J.**, **Gijselinck,I.**, Dillen,L., Van Langenhove,T., Sieben,A., Martin,J-J., **Cruts,M.**, Van Broeckhoven,C., EOD Consortium,: Geographical frequency of the FTLD-ALS causing C9Orf72 repeat expansion mutation in an extended cohort ascertained within the European Consortium on early-onset dementia. Alzheimer's & Dementia - Supplement AAIC : (2012) (I.F.: 6.373)
- Van Langenhove,T., **van der Zee,J., Gijselinck,I.**, Engelborghs,S., Vandenberghe,R., Vandenbulcke,M., De Bleecker,J., Sieben,A., Ivanoiu,A., Deryck,O., Willems,C., Martin,J-J., Michotte,A., Santens,P., De Jonghe,P., Cras,P., De Deyn,P., **Cruts,M.**, Van Broeckhoven,C.: Distinct clinical characteristics of C9orf72 expansion carriers compared to GRN, MAPT and non-mutation carries in a Flanders-Belgian FTLD cohort. International Conference on Frontotemporal dementia, Manchester, UK, September 5-7 (2012) Dementia and Geriatric Cognitive Disorders 34(S1): 226
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# Administrative Report

## Honors, prizes & awards

### *Prizes*

- **Cruts M.**: Medical Foundation Queen Elisabeth (GSKE), Solvay Prize, May 15, 2012

### *Travel awards*

- **Gijselinck I.**: Alzheimer's Association International Conference (AAIC) 2012 Travel Fellowship Committee, AAIC Travel Award for participation to an international meeting: Alzheimers's Association International Conference 2012, Vancouver, British Columbia, July 14-19, 2012
- **Gijselinck I.**: Research Foundation-Flanders (FWO), FWO Travel Award for participation to an international meeting: Alzheimer's Association International Conference 2012, Vancouver, British Columbia, July 14-19, 2012
- **van der Zee J.**: Alzheimer's Association International Conference (AAIC) 2012 Travel Fellowship Committee, AAIC Travel Award A for participation to an international meeting: Alzheimer's Association International Conference 2012, Vancouver, British Columbia, July 14-19, 2012

## Presentations

### *Invited lectures*

*International*

- **Cruts M.**: 'Weeding Mendel's Garden: Can We Hoe Dubious Genetic Associations?', Alzforum Webinar Panel Discussion, July 31, 2012

*National*

- **Janssens J.:** "Visualising angiogenesis in the context of frontotemporal lobar degeneration" Bio Imaging Lab core day, University of Antwerp, Antwerp, Belgium, October 1, 2012
- **van der Zee J.:** "Recent advances in Genetics of dementia", Interuniversity neurology course, Université Libre de Bruxelles - Hôpital Erasme, Brussels, Belgium, June 16, 2012

### *Oral presentations – slide sessions*

*International*

- **Philtjens S.:** "Identification of novel causal genes for frontotemporal lobar degeneration using whole genome sequencing", 8th International Conference on Frontotemporal Dementias, Manchester, UK, September 5-7, 2012

- **van der Zee J.:** "A European consortium for high-profile translational research on early-onset dementia – a prevalence study of the FTLD-ALS causing C9orf72 repeat expansion mutation in an extended European cohort", American Academy of Neurology 64th Annual Meeting 2012, New Orleans, LA, USA, April 21-28, 2012
- **van der Zee J.:** "Geographical frequency of the FTLD-ALS causing C9Orf72 repeat expansion mutation in an extended cohort ascertained within the European Consortium on early-onset dementia", Alzheimer's Association International Conference 2012, Vancouver, British Columbia, July 14-19, 2012
- **Van Langenhove T.:** "The clinical presentation of *C9Orf72*-associated frontotemporal lobar degeneration in an extended Flanders-Belgian cohort", Alzheimer's Association International Conference 2012, Vancouver, British Columbia, July 14-19, 2012

*National*

- **Philtjens S.:** "Identification of novel genes for frontotemporal lobar degeneration using whole-genome sequencing", Capita Selecta in Complex Disease Analysis (CSCDA) 2012, Liège, Belgium, May 31 – June 1, 2012
- **Philtjens S.:** "In search of genetic defects in unrelated frontotemporal lobar degeneration patients using whole genome sequencing", VIB Seminar 2012, Blankenberge, Belgium, April 18-20, 2012

## *Poster presentations*

*International*

- **Gijselinck I.:** "Genomic characterization of the *C9Orf72* promoter repeat in FTLD and ALS patients", Alzheimer's Association International Conference 212, Vancouver, British-Columbia, July 14-19, 2012
- **Gijselinck I.:** "Genomic characterization of the *C9orf72* repeat region associated with FTLD and ALS", 8th International Conference on Frontotemporal Dementias, Manchester, UK, September 5-7, 2012
- **Janssens J.:** "Overexpression of human p.M337V TDP-43 in mice worsens disease features of ALS/FTLD compared to wild-type mice", 8th International Conference on Frontotemporal Dementias, Manchester, UK, September 5-7, 2012
- **Janssens J.:** "Overexpression of human p.M337V TDP-43 in mice worsens disease features of ALS/FTLD compared to wild-type mice", 12th Eibsee Meeting on Cellular Mechanisms of Neurodegeneration, Grainau, Germany, November 21-24, 2012
- **Kleinberger G.:** "Reduced secretion and altered proteolytic processing caused by missense mutations in progranulin", 8th International Conference on Frontotemporal Dementias, Manchester, UK, September 5-7, 2012
- **Philtjens S.:** "Identification of novel causal genes for frontotemporal lobar degeneration using whole genome sequencing", 8th International Conference on Frontotemporal Dementias, Manchester, UK, September 5-7, 2012
- **Philtjens S.:** "In search of genetic defects in unrelated frontotemporal lobar degeneration patients using whole genome sequencing", European Society of Human Genetics (ESHG2012), Nürnberg, Germany, June 23-26, 2012
- **van der Zee J.:** "A Pan-European study of the *C9orf72* expansion associated with FTLD and ALS", 8th International Conference on Frontotemporal Dementias, Manchester, UK, September 5-7, 2012
- **van der Zee J.:** "A Pan-European study of the pathological C9orf72 hexanucleotide ($G_4C_2$) expansion associated with frontotemporal lobar degeneration and amyotrophic lateral sclerosis", American Society of Human Genetics (ASHG) Annual Meeting, San Francisco, November 6-10, 2012
- **Van Langenhove T.:** "Distinct clinical characteristics of *C9orf72* expansion carriers compared to GRN, MAPT and non-mutation carriers in a Flanders-Belgian cohort", 8th International Conference on Frontotemporal Dementias, Manchester, UK, September 5-7, 2012

*National*

- **Gijselinck I.:** "Genomic characterization of the *C9orf72* promoter repeat in FTLD and ALS patients", VIB Seminar 2012, Blankenberge, Belgium, April 18-20, 2012
- **Janssens J.:** "Overexpression of mutant human TDP-43 in mice worsens disease features of ALS/FTLD compared to wild-type human TDP-43 overexpression mice", VIB Seminar 2012, Blankenberge, Belgium, April 18-20, 2012
- **Van Langenhove T.**: "Phenotypic presentation of C9orf72-associated frontotemporal lobar degeneration", VIB Seminar 2012, Blankenberge, Belgium, April 18-20, 2012

## *Societal activities*

- **J. van der Zee:** Lay public presentation: "Dementie en het genetisch onderzoek", Davidsfonds, Ieper, Belgium, March 27, 2012
- **J. van der Zee:** Lay public presentation: "De Ziekte van Alzheimer, nieuwe wetenschappelijke inzichten rond dementie", Beurs 55+, Deurne, Belgium, May 30, 2012
- **J. van der Zee:** Lay public presentation: "Wetenschappelijk onderzoek naar Dementie", Openbare ouderenraad, Deurne, Belgium, October 15, 2012

Progress report of the research group of

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# Developmental origin of multiple defects of the nervous systems in Mowat-Wilson syndrome and its new insights for normal embryonic and adult neurogenesis

## Introduction to Mowat-Wilson syndrome (MWS)

Mutations in the *ZFHX1B* (*SIP1, ZEB2*) gene (chr2q22) cause Mowat-Wilson syndrome (MWS, MIM# 235730; www.omim.org) [1-3], a single-gene disorder characterized by various malformations that do not appear all in every MWS patient. Defects occur in the central nervous system (CNS**)** [mental retardation, delayed motor development, absence of corpus callosum, microcephaly, occurrence of seizures and epilepsy] and combine with developmental defects in the neural crest cell (NCC) lineage [cranio-facial abnormalities, Hirschsprung disease (HD)] and with a wide and heterogeneous spectrum of other anomalies. The latter include genital anomalies, eye defects, and in few patients heart defects (including tetralogy of Fallot, septal defects, patent ductus arteriosis, pulmonary arterial sling), cleft palate, sensorineural deafness and other related defects like those caused during dorsal root ganglion development [4, 5; see also Q.E.M.F. report 2011], which cause some MWS patients to be less sensitive to pain [6]. Analysis of about 220 MWS patients thus far has shown that full genomic deletion of the *ZFHX1B* locus occurs in roughly 20% of known cases. The remaining near-80% of *ZFHX1B* known mutations creates frameshift mutations, and haplo-insufficiency has been postulated as the major cause of the wide variety of symptoms in MWS. Only very few missense mutations (3 published, and 4 other ones known in the field) affecting the function of a domain of the multi-domain ZFHX1B protein are known [for a recent review on the spectrum of mutations in MWS patients, see 7].

### Some of our previous work on Sip1

Our lab was the first to discover Sip1 (Smad-interacting protein-1) by virtue of its binding to the MH2 domain of Smad proteins [8]. Sip1 was found to bind to the TGFβ/Nodal/Activin Smads2/3 and the BMP-Smads1/5/8 in ligand-stimulated cells only, although many of Sip1's functions may be Smad-independent and hence underpin multiple mechanisms of action of Sip1 [for a discussion, see 9]. Sip1 represses target gene transcription through binding with each of its two zinc finger clusters to a separated repeat of CACCT(G) – or in fewer cases CACANNT(G) – in regulatory regions of genes [10]. Full-length Sip1 binds to the co-repressor CtBP [11] and the chromatin-remodeling co-repressor complex NuRD [12]. It can become an activator as well by binding to P300/PCAF [13]. Sip1's levels are under control of miRNAs, including in epithelial-mesenchymal transition, which is relevant to invasive properties of epithelial-derived tumor cells [14-17]. Similar control circuits involving Sip1 and its related protein δEF1 (also named Zeb1 and Zfhx1a) have been proposed to operate in cancer stem cells [18].

Our studies in our *Sip1* conventional knockout mice showed that these die early in postimplantation embryogenesis, i.e. at ~E9, and display severe defects in the neural plate, neural crest and somitogenesis [19, 20]. Our work with *conditional Sip1* knockout mice (using Wnt1-Cre, which is active in premigratory and migratory NCC) suggests that the HD and facial malformation have their origin in defects in NCC [21]. Our more recent studies, focusing on brain development - and more recently neurogenesis in the injured brain of adult mice [Stappers, Seuntjens and Huylebroeck, *unpublished results*] - addressed Sip1 function in the embryonic and early postnatal CNS, particularly in the hippocampus and the brain cortex [22, 23]. This

work revealed new Sip1-controlled mechanisms emanating from postmitotic neurons in cell fate determination of progenitor cells in the normal embryonic brain cortex.

**Work performed in year 2 with the support of the Q.E.M.F.**

Our work performed in the context of the Q.E.M.F. funding mainly continues to use a combination of studies with various conditional *Sip1* knockout mice and tissue/organ explants thereof, and cell culture. The publications resulting from work that included year 2 of the Q.E.M.F. project are listed below in a separate section. The major focus is to explain in detail the embryonic origin of major clinical signs of MWS as well as carefully document newly found defects in some of our new mouse models, which include strong indications for a dual mode of function of Sip1 (as repressor and activator of gene transcription, including in interpretation of TGFβ family signaling). We had significant progress in our studies of Sip1 in the embryonic ventral forebrain, where we investigate the molecular mechanisms underlying Sip1's essential functions that regulate the tangential migration of GABAergic interneurons, which relates to seizures and epilepsy seen in MWS patients [**van den Berghe *et al.*, 2013**; **McKinsey *et al.*, 2013**]. We have also found a new role for Sip1 in motor neurons in the spinal cord, *i.e.* in Onecut family transcription factor controlled visceral motor neurogenesis [**Roy *et al.*, 2012**], and in myelination in the CNS [**Weng *et al.*, 2012**]. Other Q.E.M.F. supported work was in the area of human genetics, where our team did essential experiments on gene expression analysis in the brain cortex in the context of Nicolaides-Baraitser syndrome, which displays CNS defects [**Van Houdt *et al.*, 2012**]. In addition, we studied brain cortex developmental defects in *Bmp7* knockout mice [**Segklia *et al.*, 2012**].

**1. Sip1 is part of anti-BMP/anti-Wnt control mechanisms that are crucial for the differentiation of oligodendrocyte precursor cells into myelinating cells: possible impacts for de/re-myelination studies and for MWS patients**

Myelination in the CNS is essential for proper brain function. The molecular mechanisms by which oligodendrocytes (OGs) coordinate signals that control the myelinogenic program in the CNS remain poorly understood. Sip1 is present in Schwann cells accompanying the limb motor axons in the mouse embryo [Van de Putte and Huylebroeck, *unpublished results*]. This prompted us to engage in studies to document the role of Sip1 in myelinogenesis and as BMP-Smad activity modulator in this process. The bHLH-type TFs Olig1 and Olig2 promote myelination, whereas active BMP and Wnt/β-catenin signaling inhibit myelination. Olig1/2 were found to directly activate transcription of the *Sip1* gene in oligodendrocyte cultures. In addition, *Sip1* is direct target gene for these Olig TFs. In Olig1 KO mice, Sip1 mRNA levels are strongly downregulated in myelinating cells of the CNS spinal cord as compared with control mice. To assess the role of Sip1 in the oligodendrocyte lineage (OG), it was decided to generate with the team of R. Lu (Dallas) OG-lineage specific *Sip1* knockout mice (using Olig1-Cre developed by the Lu team). Such *Sip1* knockout mice develop generalized tremors, hindlimb paralysis and seizures from postnatal week 2. Sip1 deletion was found not to affect OG precursor cells (OPCs) but to be required for their maturation and myelination. Sip1 was found to activate the expression of crucial myelination-promoting factors normally inhibited by Wnt/β-catenin signaling, while it inhibits the myelination-inhibiting BMP signaling by antagonizing the activity of activated BMP-Smads at the level of Smad-regulated promoters of myelination-relevant genes within the same cells.

Among OG-specific target genes of Sip1 in the CNS, Smad7 was identified (using ChIP on the Smad7 promoter) as a candidate direct target gene for Sip1. Further work documented that (Sip1-induced) Smad7 is required for OG differentiation and, downstream of Sip1, and like Sip1, promotes myelination by blocking the BMP and Wnt/β-catenin signaling. In the report 2011 we announced a set of ongoing

additional experiments, which meanwhile resulted in the following observations: Smad7 overexpression can rescue the differentiation defects of Sip1 mutant OPCs, which means that Smad7 acts downstream of Sip1. Also, Smad7 was found required and sufficient for negatively regulating Wnt/β-catenin signaling that inhibits OG myelination. Overexpression of Smad7 alone or Smad7 combined with Smurf1 in rat OPCs decreased stabilized β-catenin levels. Indeed, the Smad7-Smurf complex targets and degrades TGFβ/BMP receptors by ubiquitination, thereby attenuating TGFβ/BMP.

So, this study [**Weng *et al.*, 2012**] on Sip1 identified two new candidate mediators for myelin repair, *i.e.* Sip1 and Smad7, which can now be further studied in other mouse models, including studies of remyelination, but also demyelinating disease. Finally, the role of Sip1 in CNS myelination suggests that mutations in *SIP1/ZFHX1B* may contribute to white matter defects in patients with MWS. As a critical regulator of BMP (and Wnt) signaling in OG maturation, Sip1 may represent a novel node of a regulatory network that integrates different signaling pathways and transcriptional signals that govern myelinogenesis in the CNS. In addition, modulation of the Smad signaling pathway may provide a future effective means to promote brain repair in patients with demyelinating diseases (and likely other neurological disorders) of the CNS.

**2. Sip1 co-regulates visceral motor neuron differentiation in Onecut dependent motor neuron subtype diversification in the spinal cord**

In our team, we had developed a Brn4Cre;Sip1 conditional mouse model for studies of Sip1 in neurogenesis in different embryonic CNS regions, including the spinal cord [Debruyn, Seuntjens and Huylebroeck, *unpublished results*]. Colleague F. Clotman (UCL, Brussels), in studies in the spinal cord of TFs of the Onecut class, used our anti-Sip1 antibodies to document the presence of Sip1 in the spinal cord and found that this antibody labels a specific set of motor neurons in the spinal cord. Subsequent work addressing Onecut and Sip1 functions in motor neuron subtype specification identified yet another function of Sip1 in the CNS [**Roy *et al.*, 2012**].

During development, spinal motor neurons (MN) diversify into a variety of subtypes specifically dedicated to the control of particular sets of skeletal muscles or visceral organs. MN diversification depends on the coordinated action of the transcription factor Isl1, which is critical for MN survival and fate determination. However, how these regulators cooperate to establish each MN subtype remains poorly understood. Using phenotypic analyses of single or compound mutant mouse embryos combined to gain-of-function experiments in chick embryonic spinal cord, it was demonstrated first that the Onecut family TFs critically regulate MN subtype diversification during spinal cord development. Onecut factors do that by directly stimulating *Isl1* expression in specific MN subtypes. These factors are therefore required to maintain Isl1 production at the time of MN diversification. In the absence of Onecut factors, major alterations of MN fate decision are observed. They are characterized by the conversion of somatic MN to visceral MN at the thoracic levels of the spinal cord and of medial MN to lateral MN in the motor columns that innervate the limbs [**Roy *et al.*, 2012**].

It is this aspect of the study that identified Sip1 as a novel developmental regulator of visceral MN differentiation as well. Indeed, the absence of Sip1 in prospective visceral MN at E10.5 in *Hnf6/Oc2* KO embryos suggested that Onecut factors control the onset of *Sip1* reactivation in differentiating MN. Such requirement for OC factors in *Sip1* expression seemed to be transient, as Sip1 was present in *Hnf6/Oc2* KO visceral MN at E12.5. To gain more insight into possible function of Sip1 during visceral MN differentiation and to assess whether delayed *Sip1* reactivation may contribute to the visceral MN phenotype in *One-cut* mutant embryos, MN development in our Brn4Cre;Sip1 mutant embryos

[Debruyn, Seuntjens and Huylebroeck, *unpublished results*] was studied. Importantly, in these, *Sip1* was deleted in the differentiating neurons but not in neural progenitors. At E10.5, the MN progenitor domain was not altered in the absence of Sip1, as demonstrated by a normal number and distribution of Olig2-positive cells. Newly-born MN were generated in normal amount and properly distributed as evidenced by quantification of Isl1 or Hb9-positive cells. In addition, the expression of the visceral MN marker *Foxp1* was not modified. Hence, we concluded that Sip1 is not required for MN generation and identity consolidation.

At E12.5, the somatic MN were present in normal numbers and displayed proper columnar organization and, in contrast, a strong reduction in the number of PGC MN was observed. However, the expression of visceral MN markers, including *Isl1*, was preserved. This reduction of visceral MN was not due to increased apoptosis in the spinal cord of Brn4Cre;Sip1 mutant embryos at E12.5. It was also not caused by fate conversion of visceral MN into V2a interneurons, as the numbers of Chx10-positive V2a cells were normal.

Taken together, these observations demonstrate that the presence of Sip1 between E10.5 and E12.5 is required in this area of the spinal cord for the production of proper amounts of visceral MN. In addition, Sip1 seems to have effects opposite to Onecut factors that act here. Therefore, the stimulation of *Sip1* expression by OC factors in newly-born MN might provide an additional feedback mechanism to adjust visceral MN production.

**3. Sip1 is essential for directed migration of GABAergic interneurons in the embryonic forebrain; Sip1 loss causes these cortical neurons to switch into a striatal interneuron-like fate**

As mentioned in the report 2011, we have extended studies on Sip1 in the basal ganglia of the ventral telencephalon (VT), in GABAergic interneurons (INs). These are generated by the ganglionic eminences (GEs, the medial, lateral and caudal GEs), with the medial GE generating most of the INs destined for the cortex. Directional tangential migration (which starts in the mouse at E11.5 reaching a peak at E16.5) to the cortex is along 3 well-documented routes (which avoid the striatum in the VT). Sets of intrinsic and extrinsic factors provide cues whose identity may overlap with those known from axon guidance of non-cortical neurons at other sites in the CNS. A lot of questions on the precise actors and their regulation in migration and guidance of these cortical INs remain unanswered.

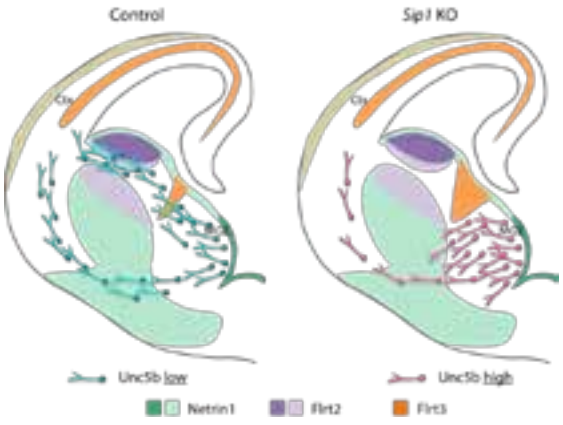


**Figure 1. Interneuron migration defect in the absence of Sip1.**
In wild type telencephalon, cortical interneurons origin in the MGE and migrate through the LGE while avoiding the striatal area. In the absence of Sip1, the levels of the receptor Unc5b, sensing the repulsive cues Netrin1, Flrt2 and Flrt3, increase. This causes the Sip1KO cortical interneurons to deviate from their path and prevent their entrance into the cortical field.

Strong Sip1 staining is found in the SVZ of the lateral GE (LGE), while weaker staining is seen in all cells of the MGE, with levels increasing in migrating INs. We used 4 different Cre-based approaches for making the respective KO mice. Using these models for Sip1, *in vivo* and *in vitro* analyses and transcriptomics

using Sip1-deficient, Cre-activated GFP-positive sorted (by FACS) forebrain cells, followed by RNA-Seq analysis, it is clear that Sip1 is critical for migration of cortical GABAergic INs. All combined data strongly suggest that Sip1 (in a cell-autonomous fashion) is crucial for interpretation of Netrin family guidance cues (many of which are upregulated in the absence of Sip1) and not for their migratory capacity *per se* (see Figure 1). The last part of this study was to validate the aberrant modulation in Sip1-deficient forebrain of a number of Netrin family proteins and their receptors, as suggested by the RNA-Seq data. For this, our team used focal electroporation (FEP) in organotypic brain slices made from E13.5 wild-type, *Sip1*$^{flex7/flex7}$ and *Sip1* conditional KO embryos. These slices were injected/electroporated with vectors either encoding a shRNA or cDNA. After FEP, slices were cultured for up to 3 days and subsequently analyzed via confocal microscopy, and the migration of the traceable cells quantified. Based on these experiments, wherein it was tried to mimic the Sip1 mutant phenotype by overproduction of *e.g.* Netrin1 and Unc5b (both upregulated in the mutant brains) in wild-type MGEs via FEP, we concluded that increased Unc5b in the MGE of wild-type brain slices disturbs IN migration to the neocortex, whereas reducing Unc5b levels in *Sip1* KO brain slices and brains rescued the migration defect. Our results thus reveal that Sip1, through tuning of Unc5b levels, is essential for interneuron guidance [**van den Berghe *et al.*, 2013**].

In an additional study, the team of J. Rubenstein confirmed that Sip1 is required in the MGE, and demonstrate that it acts directly downstream of *Dlx1 and Dlx2* to generate cortical INs that express *Cxcr7*, *MafB* and *cMaf*. In the absence of Sip1, *Nkx2-1* expression is not repressed, and cells that ordinarily would become cortical interneurons appear, based on a limited number of marker genes, to transform towards a subtype of GABAergic striatal interneurons [**McKinsey *et al.*, 2013**].

This work in these conditional knockout mouse models also revealed a relation to seizures and epilepsy observed in the majority of MWS patients.

### 4. Bmp7 Regulates the survival, proliferation, and neurogenic properties of neural progenitor cells during corticogenesis in the mouse

Our work on Smad-interacting proteins (SIPs), including Sip1, also addressed how these SIPs function in TGFβ family signaling interpretation within cells, but also addresses in a few studies in the nervous systems of mouse embryos which ligands (such as Bone Morphogenetic Proteins, BMPs) and Smads (such as the BMP-Smads Smad1 and Smad5, in collaboration with An Zwijsen, Leuven) act upstream of these SIPs.

BMPs are considered important regulators of neural development. However, results mainly from a wide set of in vitro gain-of-function experiments are conflicting since these show that BMPs can act either as inhibitors or promoters of neurogenesis. In 2012, we reported a specific and non-redundant role for BMP7 in cortical neurogenesis *in vivo* using KO mice. Bmp7 is produced in regions adjacent to the developing cortex, *i.e.* in the hem, meninges, and choroid plexus, and can be detected in the cerebrospinal fluid. Bmp7 deletion results in reduced cortical thickening, impaired neurogenesis, and loss of radial glia attachment to the meninges. Subsequent in vitro analyses of E14.5 cortical cells revealed that lack of Bmp7 affects neural progenitor cells, evidenced by their reduced proliferation, survival and self-renewal capacity. Addition of BMP7 was able to rescue these proliferation and survival defects. In addition, at the developmental stage E14.5 Bmp7 was also required to maintain Ngn2 expression in the subventricular zone. These data demonstrate a novel role for Bmp7 in the embryonic mouse cortex: Bmp7 nurtures radial glia cells and regulates fundamental properties of neural progenitor cells that subsequently affect Ngn2-dependent neurogenesis [**Segklia *et al.*, 2012**].

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## Manuscripts submitted in 2012 and published in 2012 and early 2013 with support from the q.e.m.f. (listed in chronological order)

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## Invited lectures where research supported by the q.e.m.f. was presented and the support was acknowledged

- Dec. 6, 2012: DH: Max-Planck Institute for Molecular Biomedicine, Dept for Cell and Developmental Biology, Münster, Germany (Dr. H. Schöler)
- Nov. 23, 2012: DH: Swiss Center for Regenerative Medicine, Institute for Oral Biology, University Zurich, Switzerland (Dr. D. Graf, T. Mitsiadis)
- Jun. 19-23, 2012: DH: 9th International Conference on Bone Morphogenetic Proteins, Lake Tahoe, CA, USA (Org.: A.H. Reddi) (invited speaker, chair of session)
- Apr. 17, 2012: DH: Seminar at Dept Biological Sciences and Biotechnology, Tsinghua University, Beijing, China (Dr. Y-G. Chen)
- Mar. 9, 2012: DH: Seminar at the Institute for Chemistry and Biochemistry, Freie Universität Berlin, Germany (Dr. P. Knaus)

## Defended PhD thesis with work supported by the Q.E.M.F.

- PhD thesis of **Veronique van den Berghe**, Lab. Molecular Biology (CELGEN): Conditional knockout mouse models identify Sip1/Zeb2 as a cell-autonomous transcription factor in GABAergic interneurons of the embryonic forebrain (promoter: D. Huylebroeck; co-pomoter: E. Seuntjens; public defense Dec. 10, 2012)

Progress report of the research group of

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# Characterization of Human Sleep/Wake Regulation using Multimodal Functional Imaging in Populations Stratified on the Polymorphism of *PERIOD3* Gene

## 1 Introduction

This year was mainly devoted to analysis of the fMRI study conducted last year in normal volunteers stratified according to their genotype for PER3 gene and who were submitted to a 42-hour constant routine. Preliminary results are more than encouraging and were submitted as abstracts to the forecoming SLEEP meeting.

In the course of designing this experiment, we investigated an attentional task, which eventually turned out not to be adapted to our purposes.

The Attention Network Test (ANT) is deemed to assess the alerting, orientating and executive components of human attention. Capitalizing on the opportunity to investigate three facets of attention in a single task, we used functional magnetic resonance imaging (fMRI) to assess the effect of sleep deprivation (SD) on brain responses associated with the three attentional components elicited by the ANT. Twelve healthy volunteers were scanned in two conditions 1 week apart, after a normal night of sleep (rested wakefulness, RW) or after one night of total sleep deprivation. Sleep deprivation was associated with a global increase in reaction times, which did not affect specifically any of the three attention effects. Brain responses associated with the alerting effect did not differ between RW and SD. Higher-order attention components (orientating and conflict effects) were associated with significantly larger thalamic responses during SD than during RW. These results suggest that SD influences different components of human attention non-selectively, through mechanisms that might either affect centrencephalic structures maintaining vigilance or ubiquitously perturb neuronal function. Compensatory responses can counter these effects transiently by recruiting thalamic responses, thereby supporting thalamocortical function.

**Muto V, Shaffii-le Bourdiec A, Matarazzo L, Foret A, Mascetti L, Jaspar M, Vandewalle G, Phillips C, Degueldre C, Balteau E, Luxen A, Collette F, Maquet P (2012) Influence of acute sleep loss on the neural correlates of alerting, orientating and executive attention components. J Sleep Res 21:648-658.**

We also published a follow up study of our recent Science paper on extreme chronotypes, using a different cognitive task.

Human morning and evening chronotypes differ in their preferred timing for sleep and wakefulness, as well as in optimal daytime periods to cope with cognitive challenges. Recent evidence suggests that these preferences are not a simple by-product of socio-professional timing constraints, but can be driven by inter-individual differences in the expression of circadian and homeostatic sleep-wake promoting signals. Chronotypes thus constitute a unique tool to access the interplay between those processes under normally entrained day-night conditions, and to investigate how they impinge onto higher cognitive control processes. Using functional

magnetic resonance imaging (fMRI), we assessed the influence of chronotype and time-of-day on conflict processing-related cerebral activity throughout a normal waking day. Sixteen morning and 15 evening types were recorded at two individually adapted time points (1.5 versus 10.5 hours spent awake) while performing the Stroop paradigm. Results show that interference-related hemodynamic responses are maintained or even increased in evening types from the subjective morning to the subjective evening in a set of brain areas playing a pivotal role in successful inhibitory functioning, whereas they decreased in morning types under the same conditions. Furthermore, during the evening hours, activity in a posterior hypothalamic region putatively involved in sleep-wake regulation correlated in a chronotype-specific manner with slow wave activity at the beginning of the night, an index of accumulated homeostatic sleep pressure. These results shed light into the cerebral mechanisms underlying inter-individual differences of higher-order cognitive state maintenance under normally entrained day-night conditions.

**Schmidt C, Peigneux P, Leclercq Y, Sterpenich V, Vandewalle G, Phillips C, Berthomier P, Berthomier C, Tinguely G, Gais S, Schabus M, Desseilles M, Dang-Vu T, Salmon E, Degueldre C, Balteau E, Luxen A, Cajochen C, Maquet P, Collette F (2012) Circadian Preference Modulates the Neural Substrate of Conflict Processing across the Day. PLoS One 7:e29658.**

Otherwise, we published the latest analyses of EEG/fMRI data, on the characterization of spontaneous brain activity during normal human sleep and anesthesia

Consciousness is reduced during NREM sleep due to changes in brain function which are still poorly understood. We tested the hypothesis that impaired consciousness during NREM sleep is associated with an increased modularity of brain activity. Cerebral connectivity was quantified in resting-state functional magnetic resonance imaging times series acquired in thirteen healthy volunteers during wakefulness and NREM sleep. The analysis revealed a modification of the hierarchical organization of large-scale networks into smaller independent modules during NREM sleep, independently from EEG markers of the slow oscillation. Such modifications in brain connectivity, possibly driven by sleep ultra-slow oscillations, could hinder the brain's ability to integrate information and account for decreased consciousness during NREM sleep.

**These results are now published : Boly M, Perlbarg V, Marrelec G, Schabus M, Laureys S, Doyon J, Pelegrini-Issac M, Maquet P, Benali H (2012) Hierarchical clustering of brain activity during human nonrapid eye movement sleep. Proc Natl Acad Sci U S A 109:5856-5861.**

The present study aimed at identifying the neurophysiological responses associated with auditory stimulation during non-rapid eye movement (NREM) sleep using simultaneous electroencephalography (EEG)/functional magnetic resonance imaging (fMRI) recordings. It was reported earlier that auditory stimuli produce bilateral activation in auditory cortex, thalamus, and caudate during both wakefulness and NREM sleep. However, due to the spontaneous membrane potential fluctuations cortical responses may be highly variable during NREM. Here we now examine the modulation of cerebral responses to tones depending on the presence or absence of sleep spindles and the phase of the slow oscillation. Thirteen healthy young subjects were scanned successfully during stage 2-4 NREM sleep in the first half of the night in a 3 T scanner. Subjects were not sleep-deprived and sounds were post hoc classified according to (i) the presence of sleep spindles or (ii) the phase of the slow oscillation during (+/-300 ms) tone

delivery. These detected sounds were then entered as regressors of interest in fMRI analyses. Interestingly wake-like responses - although somewhat altered in size and location - persisted during NREM sleep, except during present spindles (as previously published in Dang-Vu et al., 2011) and the negative going phase of the slow oscillation during which responses became less consistent or even absent. While the phase of the slow oscillation did not alter brain responses in primary sensory cortex, it did modulate responses at higher cortical levels. In addition EEG analyses show a distinct N550 response to tones during the presence of light sleep spindles and suggest that in deep NREM sleep the brain is more responsive during the positive going slope of the slow oscillation. The presence of short temporal windows during which the brain is open to external stimuli is consistent with the fact that even during deep sleep meaningful events can be detected. Altogether, our results emphasize the notion that spontaneous fluctuations of brain activity profoundly modify brain responses to external information across all behavioral states, including deep NREM sleep.

**Schabus M, Dang-Vu TT, Heib DP, Boly M, Desseilles M, Vandewalle G, Schmidt C, Albouy G, Darsaud A, Gais S, Degueldre C, Balteau E, Phillips C, Luxen A, Maquet P (2012) The Fate of Incoming Stimuli during NREM Sleep is Determined by Spindles and the Phase of the Slow Oscillation. Frontiers in neurology 3:40.**

We also wrote a review paper about functional imaging of human sleep:

The activity patterns adopted by brain neuronal populations differ dramatically between wakefulness and sleep. However, these vigilance states are not independent and they reciprocally interact. Here, we provide evidence that in humans, regional brain activity during wakefulness is influenced by sleep regulation, namely by the interaction between sleep homeostasis and circadian signals. We also show that, by contrast, regional brain activity during sleep is influenced by the experience acquired during the preceding waking period. These data reveal the dynamic interactions by which the succession of vigilance states support normal brain function and human cognition.

**Jedidi Z, Rikir E, Muto V, Mascetti L, Kusse C, Foret A, Shaffii-Le Bourdiec A, Vandewalle G, Maquet P (2012) Functional neuroimaging of the reciprocal influences between sleep and wakefulness. Pflugers Arch 463:103-109.**

Progress report of the research group of

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# Unravelling the roles of lysine acetylation in neural development

## Backround

The goal of our proposal is to elucidate new fundamental mechanisms that regulate neurogenesis in the developing nervous system with a focus on the cerebral cortex and the inner ear. In these structures, the generation of mature cells requires a tight coordination of multiple cellular activities including specification, cell cycle exit, migration and differentiation whose achievement relies on the implementation of transcriptional and post-transcriptional/translational events. Indeed, reversible post-translational modifications (PTM) of proteins play pivotal roles for the establishment of the nervous system (Creppe et al., 2009; Zhao et al., 2008). Numerous PTM have been identified among which some are responsible for the addition of functional groups (e.g. phosphorylation, addition of phosphate group), the addition of proteins or peptides (e.g. ubiquitination, addition of ubiquitin proteins), the modification of the chemical nature of amino acids (e.g. citrullination, conversion of arginine to citrulline), or for structural change (e.g. formation of disulfide bridges, covalent linkage between two cysteine amino acids). These chemical modifications occur after translation and regulate the activity, stability, localization or function of proteins. Lysine side chains of proteins are subjected to different rivalling and reversible PTM, including acetylation, methylation, ubiquitination, sumoylation or ADP-ribosylation (Merrick and Duraisingh, 2007). In vertebrates, lysine acetylation sites are as conserved as those in phosphorylated proteins, suggesting a selective pressure to maintain this protein modification. Recent data also indicate that such modification occurs on almost 2000 proteins (Choudhary et al., 2009), which is close to the size of the phosphoproteome. Although this PTM was until recently exclusively associated with transcriptional activation (through neutralization of positive charges of core histone tails lysines (Ren and Gorovsky, 2001)), there is now growing evidences to support lysine acetylation of a broad range of non-histone proteins (Choudhary and Grant, 2004; Close et al., 2010; Kim et al., 2006). This modification is promoted by lysine acetylases (KATs) and requires acetyl-CoA as the acetyl donor. It is believed that lysine acetylation regulates the activity, localization, specific interaction as well as stability/degradation of proteins, therefore controlling a variety of cellular processes such as apoptosis, proliferation and differentiation (Spange et al., 2009). Recent studies suggest that acetylation of cytoplasmic substrates contributes to brain development (Creppe et al., 2009; Reed et al., 2006) and, that disruption of this process is associated with various progressive neurological disorders (Dompierre et al., 2007; Hempen and Brion, 1996). Although it is widely accepted that a tight interplay between lysine deacetylases (KDACs) and KATs acts antagonistically (Creppe et al., 2009) to control protein acetylation, the enzymes that catalyse such modification on non-histone proteins remain often unknown. Thus, identifying KATs and KDACs as well as proteins whose dynamic acetylation regulates neurogenesis is pivotal to better understand the development of the central nervous system and in particular the cerebral cortex and the inner ear. This fundamental knowledge will be required to develop new therapeutic strategies for neurological and hearing disorders.

## Aims of the scientific programme

The goal of this proposal is to uncover new protein substrates that undergo lysine acetylation during the development of the nervous system. For this purpose, we will use complementary approaches to identify putative candidates. Thus we will try to understand how the acetylation of specific proteins contributes to cerebral cortical neurogenesis (Aim 1) or inner ear development (Aim 2). For this purpose

we will use a combination of genetic and molecular technologies to validate their contribution to the development of these structures.

*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.*

**Aim 1: Defining how protein (de)acetylation regulates cerebral cortical neurogenesis**

The cerebral cortex contains neurons that are distributed within layers and are regionally organized into specialized areas that underlie sophisticated motor, cognitive and perceptual abilities (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 pyramidal projection neurons born in the dorsal telencephalon (Gupta et al., 2002) and, GABAergic interneurons generated in the ventral forebrain (Anderson et al., 1997). The projection neurons undergo radial migration along radial glia fibers to settle in the cortical plate, while interneurons migrate tangentially from the medial and caudal ganglionic eminences (MGE and CGE, respectively) to reach the cortical wall. More generally, the development of the cortex progresses through several stages including, neural proliferation, neuroblast migration and neuronal differentiation. Disrupting the completion of one or several of these steps often cause cortical malformations that can lead to severe learning disabilities, mental retardation and epilepsy (Bielas et al., 2004; Gupta et al., 2002). Thus, identification of new molecular pathways that promote the formation of the cortex is critical to interpret the pathological mechanisms that contribute to the onset and the progression of these disorders. Acetylation of $\alpha$-tubulin in microtubules has recently been associated with the maturation (Creppe et al., 2009) and survival of neurons (Dompierre et al., 2007) and such modification is likely to occur on various protein substrates that are required for neurogenesis. While recent works revealed the existence of hundreds of acetylated cytoplasmic and mitochondrial proteins (Choudhary et al., 2009; Kim et al., 2006), some being expressed in neurons and their progenitors, the role of such modification and the identity of the KATs and KDACs that are responsible for the (de)acetylation of these substrates often remains unknown (Choudhary et al., 2009). Elongator is a multiprotein complex composed of 6 subunits (Elp1-6), which is expressed both in the nucleus and the cytoplasm where it plays multiple functions. It promotes acetylation of histones in the nucleus and thus contributes to transcript elongation (via the KAT domain). In addition, it promotes paternal genome demethylation (via the SAM domain). In the cytoplasm, it contributes to exocytosis and tRNA modification, and it has been shown in our laboratory that its acute loss resulted in alpha tubulin acetylation defects in microtubules. This posttranslational modification contributes to the migration and differentiation of cortical projection neurons. Acetylation of MTs contributes to Mt-dependent transport by promoting the anchoring of some molecular motors. Importantly, molecular transport underlies the regulation of cell shape remodeling during neuritogenesis and neuronal migration. We thus tested the effect of Elongator depletion on the retrograde and anterograde transport of specific cargo proteins in both mouse cortical neurons in culture and instar 3 drosophila larva motoneurons in vivo. We are currently analyzing defects resulting from loss of function of Elp3 an/or Mec-17 (a potent promoter of alpha-tubulin acetylation (Akella et al., 2010)) to 1/assess individual transport phenotypes in drosophila, 2/ combine invalidation of both genes to induce a more efficient depletion of acetylation of MT. Those results will be compared to those obtained with hiPS cell-derived neurons from Familial Dysautonomia patients (collaboration with L. Studer). Those informations should help us understanding the pathological mechanisms that underlie neurodevelopmental and neurodegeneration defects in Familial Dysautonomia

***We searched for additional cytoplasmic candidate proteins that are acetylated by Elongator and that promote the development of the cerebral cortex***. For this purpose, we combined a

candidate-based approach with a proteomic screen to compare the cortical acetylome (proteome of acetylated proteins) of WT and cKO Elp3 mice. One of the candidate proteins is a connexin. We found that this connexin is enriched in the developing cerebral cortex and is massively acetylated. Western blot analyses performed on cortical extracts from Elp3 conditional knockout (Elp3lox Foxg1:Cre) E12 mouse embryos showed a dramatic reduction of the level of acetylation of the connexin. In addition, co-immunoprecipitation assays with cortical tissue extracts demonstrated an interaction between the connexin and Elp1, the scaffold subunit of the Elongator complex. Thus, this connexin is a strong candidate for acetylation by Elongator. These results have been confirmed in several mouse and human cell lines. In addition, we found that HDAC6 is a KDAC responsible for the deacetylation of this connexin. We are currently assessing the putative role of connexin acetylation in corticogenesis, focussing on the control of the interkinetic nuclear movement (INM). We performed BrdU injection followed by immunohistology to study INM kinetics. BrdU injections were performed in pregnant mice to label cycling cortical progenitors in S or G2 phases in E14.5 Elp3 cKO or WT embryos followed by sacrifice after several timings (1h or 4h for assessing S-phase or G2 phase, respectively). Our preliminary result suggests that INM is affected upon depletion of Elp3. This biological event has previously been linked to connexin expression but not its acetylation. We are currently identifying the lysine residues of Connexin 43 targeted by Elp3 with mass spectrometry on N2A cells, that expressed or not Elp3. We will perform mutations to important lysine residues by arginines. Plasmids coding for various lysine mutant forms will be engineered and delivered together with Cre-expressing vectors (to remove the endogenous Elp3) by in utero electroporation into cortical progenitors from cKO Elp3 embryos. A second aspect is the control of cortical progenitors specification and their ability to generate projection neurons. The overall phenotype of Elp3 cKO cortex is a reduced cortical plate thickness arising as a consequence of the major depletion postmitotic projections neurons. We discovered that Tbr2 is significantly impaired in Elp3 cKO cortices as compared to control. These cells are required for the production of most projection neurons dedicated to all cortical layers. Our preliminary results suggest that Elongator may control the proliferation and or specification of Tbr2 intermediate cortical progenitors whose reduction would impact on the total neuronal output and could thus explain the cortical phenotype of Elp3 cKO embryos. We are now investigating if and how Elongator controls the expression of Tbr2 in the developing cortex.

To investigate the ***role(s) of Elongator in tangential migration*** of cortical interneurons, we used an Elp3 flox; Dlx5,6 Cre-GFP mouse line (Elp3 cKO) newly generated in our laboratory. Real-time experiments on Elp3 cKO or WT MGE explants were performed on interneurons to analyse their migration as well as their cell shape modifications. After 24 hours of culture, control interneurons that have migrated out of MGE explants exhibited a polarized morphology with branched leading process. Our preliminary data indicated that interneurons that lack Elp3 expression had a significant decrease of migration velocity as well as a reduced frequency and amplitude of nuclear translocations. In addition, only 40% of Elp3 cKO migrating interneurons displayed a swelling. Real time imaging also indicated that the formation and division of growth cones (that underlie the production of new branches) were both, less frequent and less stable in Elp3 cKO interneurons, as compared to control experiments. Some of these results were confirmed in situ, cultured brain slices. Furthermore, immunolabeling of E12.5 embryo sections showed that the loss of Elp3 expression resulted in abnormal cellular shape and in a significant reduction of the number of GABAergic interneurons that entered into the cortex. This observation suggests that the conditional removal of Elp3 resulted in a tangential migration delay and supports the time-lapse results on MGE explants. Collectively, our data describe a novel role for Elp3 in the control of nucleokinesis kinetics, branching dynamics of interneurons, growth cone splitting and stability of newly formed neurites. To determine how Elp3 controls these parameters, we will combine time–lapse recording of MGE explants from Elp3 cKO GABAergic interneurons with rescue experiments. For this, we will

electroporate vectors coding for Elp3 protein that lack either the Histone acetyl transferase (HAT) or the DNA methyltransferases (SAM) domains. In order to untangle the molecular mechanisms triggered by Elp3 to control tangential migration, we will FACS MGE- and CGE-derived GFP-positive interneurons and we will perform microarray experiments and mass spectrometry analyses to identify new genes or proteins regulated by ELP3.

**Aim 2: Defining how protein (de)acetylation regulates the development of the inner ear**

The development of the inner ear involves multiple processes including proliferation (in mice, ranging from E12.5 to E14.5 in the cochlea and between E12.5 and P2 in the vestibule) and specification of progenitors (between E15.5 and P4) into hair cells, the highly specialized mechanoreceptors, and supporting cells of the sensory epithelia. Concomitantly, epithelial neuroblasts delaminate from the otic epithelium to form the neurons of the cochleo-vestibular ganglion, which innervate the otic sensory elements (Rubel and Fritzsch, 2002). In the mammalian inner ear, similarly to the central nervous system (CNS), the regenerative capacity of hair cell and/or cochleo-vestibular neurons is lost during adulthood and no functional compensation is achieved. Consequently, deafness or balance dysfunctions, commonly resulting from lesion of the hair cells and/or of the neurons of the auditory or vestibular part of the inner ear, respectively, are permanent. There are currently no treatment designed to halt or prevent the progression of hearing loss or vertigo, therefore, understanding the molecular signals that control the number of progenitors, their differentiation and their tissular organization in the inner ear is a prerequisite for developing new strategies to promote hair cell regeneration and partially restore hearing. The main objective of this part of the project is to uncover the role of lysine acetylation during the inner ear development. We first focused our attention on the role of Elongator complex.

We first unravel the temporal and spatial expression of Elp3 and Elp1, two main members of the Elongator complex. Elp1 and Elp3 mRNA transcripts have been detected in the developing inner ear and have a strictly overlapping pattern of expression. At E11.5, the first stage studied, they are present in the entire otic vesicle and absent in the surrounding mesenchyme. Later, there expression became mainly restricted to neurons in the cochlea-vestibular ganglion and to the sensory epithelium in the cochlea and the vestibule.

Using the newly created mouse line Elp3loxp/loxp, we generated FoxG1-cre conditional Elp3 knockout mice (referred to as Elp3 cKO) allowing the deletion of Elp3 in the entire otocyst at ≈E8.5. Although they were viable, these mice exhibited balance-related behavioral phenotype characterized by a tilted position of the head, circling movements, and a marked tendency to walk backwards when placed outside their cages. In addition, in the tail-hanging reflex, which normally induces a forelimb extension to reach the ground, they tended to bend ventrally and curl up their tail. More recently, we showed that these mice are deaf. Indeed, the auditory brain stem threshold were elevated in Elp3 cKO mice as compared to wild-type littermates.We also analyzed Elp3 cKO mice at the cellular level both in the sensory epithelium and the cochlea-vestibular ganglion. Preliminary results showed that the kinocilium, a specialized primary cilium, is disorganized and that the adjacent stereocilia are misaligned in Elp3 cKO mice. Taken together, these results are in favor of a role of Elp3 in planar cell polarity. In the spiral ganglion (innervating cochlear hair cells), loss of Elp3 is associated with a massive neuronal apoptosis at E14.5. There is also a conspicuous decrease of the number of fibers that innervate hair cells. In addition, numerous remaining fibers present aberrant projections towards inner hair cells. More recently, we showed that the number of synaptic ribbons, quantified after immunostainings using anti-Ctbp2/Ribeye antibody is significantly decreased at the level of inner hair cells,Altogether, these results indicate that Elp3 seems to be involved in neuronal survival and axonal guidance in the cochlea. RNA-seq study is currently performed in order to identify transcriptional targets of Elp3. A candidate approach will also be undertaken and especially we will focus our attention on Ctpb2 as previously demonstrated as an acetylable protein (Zhao et al., 2006).

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

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

The major aims of our project are to dissect out the distinct properties and identify the precise role of striatal neuronal populations and genes in motor control, movement disorders, instrumental learning and drug addiction through sub-regional ablations and optogenetic control of specific striatal neuron populations, inactivation of genes involved in motor learning and drug addiction in these neuronal subpopulations and functional characterization of genes identified in comparative gene profiles of striatopallidal and striatonigral neurons.

The work completed in 2012 thanks to the support from FMRE/GSKE is summarized below.

## 1 Deciphering the role of $D_1R$-striatonigral and $D_2R$-striatopallidal MSN of striatal subregions in motor control, instrumental learning and drug addiction.

### 1.a Effects of specific ablations $D_1R$-striatonigral and $D_2R$-striatopallidal MSN in distinct dorsal striatum subregions.

Using a transgenic $A_{2A}R$-Cre mouse strain that we developed (Durieux et al., 2009,2011) and which allowed to conditionally target the expression of a human diphteria toxin receptor (DTR) in striatopallidal neurons, our previous works showed that selective ablations of these $D_2R$-MSN can be performed in different restricted functional areas of the striatum and produce specific behavioural alterations. Indeed, $D_2R$ striatopallidal MSN ablation in the entire striatum produces permanent hyperlocomotion while restricted ablation in the ventral striatum resulted in an increase in drug reinforcement and in its much longer persistence, demonstrating that $D_2R$ striatopallidal MSN exert inhibitory functions on both locomotor control and drug reward process (Durieux et al., 2009,2011). We have further developed a parallel model allowing specific removal of the $D_1R$ striatonigral MSN (Durieux et al., 2012) by using a similar strategy and a Drd1a-Cre mice strain (Gong et al., 2007) targeting striatonigral neurons. Analysis of the resulting mice in locomotor paradigms indicate that $D_2R$ and $D_1R$ MSN inhibit and stimulate motor activity, respectively. We assessed for the respective roles of $D_1R$ striatonigral and $D_2R$ striatopallidal MSN in motor skill learning, by training mice in a motor skill learning task on an accelerating rotarod. Full ablation of $D_2R$ striatopallidal MSN resulted in early impairments, whereas mice with full ablation of $D_1R$ striatonigral MSN were unable to learn the task and displayed a permanent deficit (Durieux et al., 2012). We also showed that execution of a previously learned motor sequence is not dependent on the $D_2R$-MSN pathway while $D_1R$-neurons are still necessary for performance (Durieux et al., 2012).

The dorsal striatum is subdivided into the dorsolateral striatum, DLS, corresponding to the primate putamen, predominantly innervated by the sensorimotor cortex and the dorsomedial striatum, DMS, homologous to primate caudate nucleus, receiving projections from prefrontal and other association cortices (Graybiel, 2008). The DMS is more engaged during initial stages of motor skill learning, when the task is more dependent on attention and susceptible to interference (Luft and Buitrago, 2005) while the DLS is required for progressive skill automatization and habit learning (Yin et al., 2004, 2009). Elimination of $D_1$R-striatonigral neurons in the DMS induced a reduction in ambulation while, in contrast, DMS $D_2$R-striatopallidal neuron ablated mice displayed hyperlocomotion. This showed that the modulatory influence on locomotion observed following full ablations was recapitulated in DMS-restricted ablations, indicating that associative striatum area exerts a MSN population-dependent control over spontaneous locomotion. (Durieux et al., 2012). In the same line, we also showed that the direct pathway in the associative cortico-striatal loop (DMS) is necessary for novelty-induced exploration and that in DMS $D_2$R-neuron ablated mice, a continuous translation of sensory stimuli to locomotion lead to a state of continuous exploration/locomotion.

Evaluation of involvement of DLS and DMS $D_1$R striatonigral and $D_2$R striatopallidal neurons in motor skill learning showed that in naive animals, when task performance is more susceptible to interference and more dependent on attention (Luft and Buitrago, 2005), $D_1$R- and $D_2$R-MSNs work in concert to promote acquisition of a new motor skill. Activation of $D_1$R-MSNs in the sensorimotor striatum is required for progressive automaticity of task performance, while activation of $D_2$R-MSNs in associative striatum inhibits competing exploratory activity. During later skill learning stage, attention to action is less required and DMS $D_2$R-MSNs progressively disengage of the process while DLS $D_1$R-MSN pathway is still required for skill automatization.

Treatment of schizophrenia positive symptoms with typical neuroleptic drugs is often associated with motor side effects such as catalepsy. We evaluated involvement of each neuronal population in motor responses to neuroleptic drugs and showed that only $D_2$R-striatopallidal neuron removal selectively in the associative striatum completely abolished haloperidol–induced catalepsy, indicating that $D_2$R-antagonism in striatopallidal neurons of the associative striatum (DMS) is critical for the motor effects of haloperidol (Durieux et al., 2012). In the same line, we also showed that DMS $D_2$R-neurons necessarily contribute to the development of behavioural sensitization to amphetamine (Durieux et al., 2012), while numerous studies identified the ventral striatum as the main striatal region contributing to psychostimulants sensitization.

Altogether, our results provide direct *in vivo* experimental evidence for dissociation between neuronal subtypes and striatal subregions in the regulation of novelty or drug-induced motor responses and motor learning.

### 1.b Specific optogenetic control of $D_1$R-striatonigral and $D_2$R-striatopallidal MSN

The models described above allow a functional cell-type dissection of different striatal regions with a high spatial resolution, but are not reversible. We therefore start to develop optogenetics, that is based on the use of light-activatable proteins (“opto-”) encoded in DNA (“-genetic”) to reversibly modulate in physiological timescale, *in vivo* or *ex vivo,* the activity of genetically targeted neuronal populations in rodents. We had previously examining the feasibility of the technic *ex vivo*. Adeno-associated virus (AAV), in which expression of Channelrhododopsin-2 (ChR2) cation channel, fused with eYFP, is dependent upon Cre-recombination has been stereotactically injected for transfection into the striatum of $A_{2A}$R-Cre mice (see above and Durieux et al., 2009,2012). Experiments combining perforated patch

clamp recording and optogenetics have been carried out *ex vivo* on striatum-containing brain slices from these mice. We demonstrated that a good proportion of neurons expressed eYFP and that these neurons are selectively striatopallidal MSN since they co-expressed enkephalin. Illumination of these neurons with a blue light (470 nm) resulted in fast inward currents when recorded in voltage clamp and in the evocation of action potentials when recorded in current clamp. The validity of the technique was also demonstrated by the high-frequency reliability of the light-induced action potentials in trains. We have now started the development of the design for *in vivo* behavioural paradigms and have obtained first reliable light-induced behaviours as light-induced circling behaviour in unilaterally-injected $A_{2A}$R-Cre mice.

### 1.c Specific inactivation of NR1 in striatopallidal and striatonigral neurons

Neuroadaptation and more specifically synaptic plasticity involve several important neurotransmitter receptors and intracellular signalling cascades. Among the involved receptors, the $Ca^{2+}$ permeable glutamate NMDA receptor is a central and initial player. This has been firmly demonstrated at different excitatory synapses such as in the hippocampus (Tsien et al., 2006). The NMDA receptor seems to have key influence in the mechanisms of reward and addiction as well as in motor skill learning (Nestler, 2001). Synaptic plasticity at the corticostriatal synapses is partially dependent on these receptors in interaction with dopamine and adenosine $A_{2A}$ receptors. We have generated $A_{2A}$R-Cre:$NR1^{f/f}$ mice to specifically inactivated NR1 in striatopallidal neurons. The characterization of $A_{2A}$R-Cre/+ $NR1^{f/f}$ mice showed a selective but moderate decrease in NMDA receptor binding in the caudate-putamen and accumbens nucleus as compared to the cerebral cortex. Preliminary results showed that these mice exhibit motor dysfunctions with spontaneous hyperlocomotion and motor skill learning defects. Interestingly, these deficits are similar to those observed following the selective ablation of $D_2$R striatopallidal MSN (see above and Durieux et al., 2009,2012), suggesting that NMDA receptor is required both for learning and spontaneous motor behaviour. However, drug addiction behavioural paradigms as sensitization or conditioned place preference have not shown significant difference between control mice and $A_{2A}$R-Cre/+ $NR1^{f/f}$ mice. A recent publication demonstrated that these NR1 floxed mice (Tsien et al., 1996) have not a yield of cre recombination of 100% and that other strain of NR1 floxed mice (Dang et al., 2006) has a better recombination's yield because the LoxP sites are closer (Belforte et al., 2010). We have established collaboration with Prof. Li and have crossed his NR1 floxed mice with our $A_{2A}$R-Cre/+ mice to obtain new $A_{2A}$R-Cre/+ $NR1^{f/f}$ as well as with Drd1a-Cre/+ mice to obtain Drd1a-Cre/+ $NR1^{f/f}$. This should allow to obtain a better cell-specific NR1 inactivation in both $D_1$R striatonigral and $D_2$R striatopallidal neurons. In addition, to increase our chance of full recombination, we developed a strategy allowing to generate $A_{2A}$R-Cre/+ $NR1^{delta/f}$ and Drd1a-Cre/+ $NR1^{delta/f}$ . Finally, in order to allow the identification of neurons deficient in NR1 in brain slices for patch clamp recordings, these mice were further crossed with reporter mice (LoxP-Stop-LoxP-YFP) leading to the expression of *Yellow Fluorescent Protein* (YFP) in recombined neurons. We obtained first series of mice and showed that in a significant percentage of YFP-labelled neurons (but not in all), there is a total absence of the NMDA receptor-mediated component of the EPSC. First series of behavioural analysis have been performed on these new $A_{2A}$R-Cre/+ $NR1^{f/f}$ mice, including locomotor activity, motor skill learning, instrumental learning and novel object recognition test. Correlation of behavioural alterations with identification of neuroadaptative changes in the striatal microcircuit will be realized using patch clamp recordings and 3D-reconstruction of the recorded neurons to identify alterations in intrinsic excitability, cortico-striatal synaptic transmission and plasticity as well as cell morphology (spines density, …).

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

To gain a more complete picture of the functional diversity of MSN (Ena et al., 2011), we have previously set up protocols to purify MSN subpopulations by FACS-sorting of samples prepared from GFP-striatopallidal ($A_{2A}$R-Cre Z/EG) mice retrogradely labelled for striatonigral MSN. Gene profiles of these neurons have been obtained by micro-arrays and showed 248 striatopallidal neuron specific genes and 493 striatonigral neuron specific genes (> 2 fold differential expression). Although some genes were already known to be highly restricted to one of these subpopulations, several striatonigral neuron-specific or striatopallidal neuron-specific genes that showed a relative expression of several tens to hundred fold, were not known to be selectively expressed or even not known to be expressed in the striatum. This differential gene expression has been validated by using different techniques for a dozen of genes. Among these genes, we have selected a series of striatopallidal neuron-specific genes as NTe5, RGS5, GuaCy13A, Adk that exhibit both a high differential expression and a putative physiological relevance for further analysis using different knock-down strategies.

We validated the specific expression of the nucleotidase NTe5 in striatopallidal neurons by RT-PCR, enzymatic activity histochemistry and immunocytochemistry. NTe5 is an ecto-enzyme known to produce extracellular adenosine from 5'-AMP Since adenosine, acting at $A_1$ and $A_{2A}$ receptors, is an important neuromodulator in the central nervous system and more specifically in the striatum where $A_{2A}$ receptors are highly enriched and play crucial functional roles (Schiffmann et al., 2007), such a specific expression may have important functional impact. To assess for the involvement of this nucleotidase in behaviour, we used a global NTe5 knockout mice strain and develop striatal or striatopallidal neuron selective knock-down strategies using lentivirus-mediated small hairpin RNA. In order to analyze a striatal-dependent learning task, mice were trained in a motor skill learning task on an accelerating rotarod. This behavioral analysis on both striatal and striatopallidal neuron knock-down models as well as on the knockout mice showed that mice behave less accurately, demonstrating therefore the implication of this enzyme in striatopallidal neurons in motor learning.

To test if this deficit results from a decrease of $A_{2A}$ receptor activation as a consequence of the absence of the nucleotidase and decrease in extracellular adenosine, we quantified the catalepsy induced by the injection of the dopamine $D_2$ antagonist haloperidol, a behaviour dependent on the tonic activation of $A_{2A}$ receptor. We showed that the cataleptic response to haloperidol was significantly decreased in the KO mice compared to wild-type

RGS5 is a member of the large family of Regulators of G-protein Signalling (RGS) that are negative regulators of signalling cascades induced by proteins G. Although its role has been examined in blood vessels, very few data reported the involvement of RGS5 in brain functions apart from the fact that association study has shown a possible involvement of RGS5 in the schizophrenia symptom severity and that striatal RGS5 mRNA is regulated following amphetamine administration. We examined RGS5 knock-out mice in several paradigms to evaluate locomotor activity, motor coordination and learning, anxiety–like behavior and behavioral responses to psychostimulants (Ena et al., in preparation).
Altogether, we developed a reliable method applied on adult brain to identify and generate specific striatopallidal and striatonigral neuron gene profiles. Our approach led to the identification of new striatopallidal and striatonigral neuron-specific genes. Finally, our results highlighted the central role of an ecto-nucleotidase associated to adenosine receptors in striatal-dependent learning.

## 2 Regulation of striatal neurons excitability and corticostriatal synaptic transmission: Neuronal excitability of striatal fast-spiking interneurons deficient in parvalbumin and their synaptic connections to MSN.

Striatal neurons (MSN) excitability and corticostriatal synaptic transmission are tightly regulated both by a large series of neurotransmitters and by striatal interneurons.
Striatal fast spiking interneurons (FSI) modulate the output of the striatum by providing a powerful feedforward inhibition on striatal MSN and synchronizing their activity. Recent studies have broadened our understanding of FSI, showing that they are implicated in severe motor disorders as Parkinsonism, dystonia and Tourette syndrome. FSI are the only striatal neurons to express the $Ca^{2+}$-binding protein parvalbumin (PV). This selective expression of PV raises questions about the functional role of this $Ca^{2+}$ buffer in controlling FSI $Ca^{2+}$ dynamics, and, consequently, the FSI spiking mode and neurotransmission. Therefore, to study the functional involvement of FSI in striatal microcircuit activity and the role of PV in FSI function, we performed perforated patch recordings on EGFP-expressing FSI in brain slices from control and PV-/- mice (Orduz et al., submitted). Our results revealed that PV-/- FSI fired more regularly and were more excitable than control FSI by a mechanism in which $Ca^{2+}$ buffering seems to be linked to spiking activity as the result of the activation of small conductance (SK) $Ca^{2+}$-dependent $K^+$ channels (Orduz et al., submitted). Numerical simulations in a mathematical model could be a suitable approach to verify this hypothesis. However, so far the existing conductance-based computational models for FSI did not allow the study of the coupling between PV concentration and electrical activity. Therefore, we generated a new mathematical model for the FSI that includes apamin-sensitive SK channels and takes into account the presence of a $Ca^{2+}$ buffer (Bischop et al., 2012). We found that this modeling approach of striatal FSI fully supported our experimental results by showing, for instance, that a variation in the concentration of PV substantially modulates the intrinsic excitability of FSI (Bischop et al., 2012; Orduz et al., submitted).

To test the impact of presynaptic PV on FSI neurotransmission, FSI synaptically-connected to MSN were recorded in a double patch mode. In recordings from the post-synaptic MSN, no differences were observed, either in IPSC kinetics or in failure rate or quantal size, indicating that the general properties of this inhibitory neurotransmission were not changed by PV during single pulse protocols. However, we also examined whether presynaptic PV leads to short-term modulation of synaptic plasticity by recording paired-pulse ratios (PPR) at FSI-MSN synapses. We showed that PV deletion modified frequency-specific short-term plasticity at these FSI-MSN synapses since, in a narrow temporal window between 20 to 50 ms, they exhibit a paired-pulse depression in wild-type that is reversed to a clear paired-pulse facilitation in PV-/- mice (Orduz et al., submitted).

Altogether, our results demonstrated that in FSI, PV is crucial for the fine-tuning of the temporal responses of the FSI network and for the orchestration of MSN populations. This, in turn, may play a direct role in the generation and pathological worsening of motor rhythms.

## 3 Additional projects and collaborations based on expertise developed under the frame of this program.

We previously characterized the distribution of the basal ganglia-specific synaptic protein SV2C by showing that it is highly expressed in dopaminergic neurons, in striatal cholinergic interneurons and, at a moderate level, in both MSN subpopulations (Dardou et al., 2011). We have now investigated the implication of SV2C in both normal and pathological basal ganglia functioning. We showed that in SV2C-deficient mice ($SV2C^{-/-}$) mice, the expression of tyrosine hydroxylase mRNA in midbrain dopaminergic neurons was largely and significantly increased whilst enkephalin mRNA expression was significantly decreased in the caudate-putamen and accumbens nucleus (Dardou et al., 2012). In two models of dopaminergic denervation, SV2C mRNA expression was significantly increased in the striatum. In order to further understand the role of SV2C, we performed behavioural experiments on $SV2C^{-/-}$ mice and on knock-down mice receiving an injection of adeno-associated virus expressing SV2C miRNA specifically in the ventral midbrain. We showed that even if these modifications of SV2C expression had no impact on behaviour in open field and elevated plus maze, the specific knock-down of SV2C expression in the dopaminergic neurons completely abolished the development of a conditioned place preference induced by cocaine while the reaction to an acute drug injection remains similar in these mice compared to control mice (Dardou et al., 2012). These results suggest that SV2C is involved in normal operation of the basal ganglia network and could also be involved in system adaptation in basal ganglia pathological conditions.

In collaboration with the group of Martin Schwab from the Brain Research Institute at University of Zurich, we investigated the function of neuronal Nogo-A. Nogo-A is a membrane protein enriched in the adult central myelin, where it restricts the capacity of axons to grow and regenerate after injury but is also expressed by certain neurons, in particular during development, where its physiological function was less well understood. In the cerebellum, Nogo-A is transitorily highly expressed in the Purkinje cells (PC) during early postnatal development. By using Nogo-A-deficient ($Nogo\text{-}A^{-/-}$) mice and mice with a selective overexpression of Nogo-A in PC and patch clamp recordings as well as 3D confocal reconstruction of recorded PC, we analyzed its effect on dendritogenesis and on the formation of their main input synapses from parallel (PF) and climbing fibers (CF). PC dendritic trees were larger and more complex in $Nogo\text{-}A^{-/-}$ mice and smaller than in wild-type in Nogo-A overexpressing PC. $Nogo\text{-}A^{-/-}$ resulted in premature soma-to-dendrite translocation of CF and an enlargement of the CF territory in the molecular layer during development. Although spine density was not influenced by Nogo-A, the size of postsynaptic densities of PF-PC synapses was negatively correlated with the Nogo-A expression level. Moreover, PC patch clamp recordings revealed that Nogo-A negatively regulates the strength of synaptic transmission at the PF-PC synapse. Thus, our results demonstrated that Nogo-A is a negative regulator of PC input synapses which orchestrates cerebellar connectivity through regulation of synapse morphology and size of the PC dendritic tree. They identified Nogo-A as a putative negative controller of synaptic development, strength and structure in several brain regions.

We took part to the study of Jens Eilers and collaborators from the Carl-Ludwig Institute for Physiology at University of Leipzig on the coupling between presynaptic $Ca^{2+}$ and transmitter release and hence synaptic transmission (Schmidt et al., 2012). The coupling distance between presynaptic $Ca^{2+}$ influx and the sensor for vesicular transmitter release determines speed and reliability of synaptic transmission. We have analyzed excitatory PF to PC synapses in the cerebellum of wild-type mice and mice deficient in the $Ca^{2+}$ binding protein calretinin, specifically expressed in the granule cell and their axons (PF) (Schiffmann et al., 1999). The coupling distance was quantified by combining fluctuation analyses in

patch clamp recordings, presynaptic $Ca^{2+}$ imaging, and reaction-diffusion computer simulations. We found a coupling distance of <30 nm at these synapses, much shorter than at any other glutamatergic cortical synapse investigated to date. Our results suggest that nanodomain coupling is a general characteristic of conventional cortical synapses involved in high-frequency transmission.

We took part to the functional characterization of human embryonic (ESC) and induced pluripotent (iPSC) stem cells differentiated in functional pyramidal neurons (Espuny-Camacho et al., 2012) that have been generated and characterized by the Pierre Vanderhaeghen's lab. By first using calcium imaging, we demonstrated spontaneous calcium waves that were blocked by tetrodotoxin. We next demonstrated by performing patch-clamp recordings a clear temporal evolution in the electrophysiological properties of the cell population, with increased proportion of cells displaying spontaneous synaptic currents (EPSCs) and repetitive firing (Espuny-Camacho et al., 2012). This was corroborated at the single cell level by the observation of an increase with time in the intensity of the voltage dependent sodium current and the amplitude of action potentials. Together with other in vitro and in vivo data, these results demonstrate that human cortical neurons generated *in vitro* from ESC/iPSC can develop complex hodological properties characteristic of the cerebral cortex *in vivo*.

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Progress report of the research group of

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# Unveiling the role of the cystine/glutamate antiporter (system $x_c^-$) in hippocampal functioning, mechanisms of epilepsy and its comorbidities: a new era for future drug treatment

## I State-of-the-art and objectives

### I.1 Epilepsy and its comorbidities

As many as 6 million people in Europe suffer from active epilepsy which has major implications for healthcare but also for education, employment, independent living, mobility and relationships of these patients (Baulac and Pitkänen, 2008). Cognitive impairment and major depression are common disabilities associated with refractory epilepsy. Important contributing factors may be the overlap in synaptic plasticity mechanisms underlying memory formation and epileptogenesis (Meador, 2007) as well as abnormalities in transmitter systems (e.g. monoamines, glutamate) involved in depression and epilepsy (Jobe et al., 1999; Smolders et al., 2008; Sanacora et al., 2012). The prognosis and quality of life of a person with epilepsy varies considerably according to the type, frequency and severity of the seizures. Temporal lobe epilepsy (TLE) is one of the most common and difficult-to-treat epilepsies: up to 25-40 % of patients develop pharmacoresistance. Thus, millions of epilepsy patients continue to experience disabling seizures despite a considerable number of registered antiepileptic drugs (AEDs). The search for new AEDs with novel mechanisms of action and improved activity therefore remains highly relevant. There is also a need for innovative therapies that are not merely symptomatic (anticonvulsive), but that can prevent epilepsy (antiepileptogenic), halt its progression (disease-modification) and treats comorbid dysfunctions such as cognitive decline and/or depressive symptoms.

### I.2 The cystine/glutamate antiporter (system $x_c^-$): current knowledge on its functions in the brain

The cystine/glutamate antiporter or system $x_c^-$ is a membrane-bound $Na^+$-independent amino acid transporter that is structurally composed of a heavy chain subunit common to all amino acid transporters, 4F2, and a light chain specific subunit, xCT (Sato et al, 1999). Disturbances in functioning of brain system $x_c^-$ can have dual physiological implications. Indeed, this antiporter provides cells with cystine that is intracellularly reduced to cysteine, the rate-limiting building block of the major brain antioxidant glutathione (GSH) and inhibition of system $x_c^-$ can thus lead to increased oxidative stress. However, system $x_c^-$ is also a source of non-vesicular glutamate, possibly enhancing excitability and eventually leading to excitotoxicity, meaning that inhibition could protect the brain.

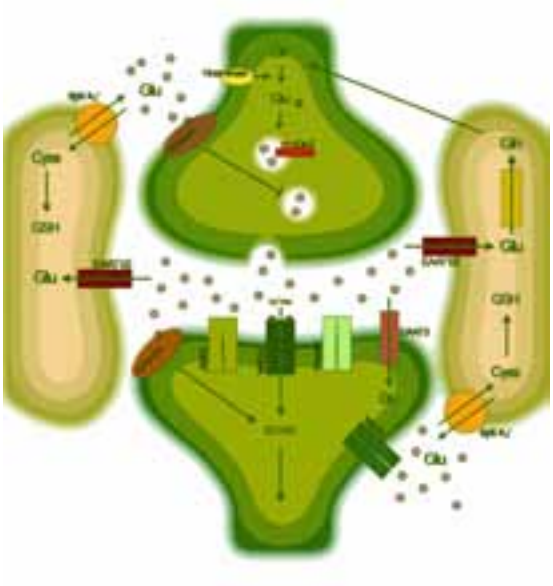

Figure 1 Key proteins of glutamate transmission

System $x_c^-$ has received considerable attention because it is upregulated in various cancers, including malignant brain glioma (Chung et al, 2005). A large body of evidence indicated that disruptions in

glutamate homeostasis and neuroadaptations in system $x_c^-$ are associated with addictive behaviours to drugs of abuse and that targeting system $x_c^-$ inhibits drug-seeking and relapse in rodents and humans (Kalivas, 2009). With regard to neurological disorders, most of the work to date has focussed on *in vitro* studies, from which it became clear that system $x_c^-$ has a pivotal function in supporting GSH biosynthesis for survival and proliferation of many cell types (Albrecht et al, 2010). However, *in vivo* studies investigating to which extent dysfunction of system $x_c^-$ affects GSH levels and oxidative stress in the brain were lacking and became recently the subject of our interest.

We first investigated whether homozygous deletion of the specific system $x_c^-$ subunit xCT ($xCT^{-/-}$ mice) affected brain GSH content and oxidative stress-related markers before looking further into animal models for neurological disorders. In a first study, we focussed on the nigrostriatal pathway and a mouse model for Parkinson's disease, known to be dependent on oxidative stress-related neuronal damage. xCT deletion did not affect striatal GSH levels, and no signs of increased oxidative stress were observed in striatum or substantia nigra of $xCT^{-/-}$ mice under physiological conditions. We did notice a decrease of 70% in striatal extracellular glutamate levels, and $xCT^{-/-}$ mice were clearly less susceptible to 6-hydroxy-dopamine-induced neurodegeneration in the substantia nigra compared to age-matched wild-types (Massie et al, 2011).

In a second study (De Bundel et al, 2011; see also GSKE report 2011), we unravelled whether system $x_c^-$ also controls hippocampal functioning in both baseline and disease conditions. Deletion of xCT had no effect on hippocampal GSH content or oxidative stress markers. With ageing $xCT^{-/-}$ mice did not display exacerbated cortical thinning, hippocampal atrophy or glial cell loss in the hippocampus. $xCT^{-/-}$ mice learned both the procedural and spatial aspects of the water maze task and displayed intact spatial reference memory. Together these results indicate that loss of system $x_c^-$ does not induce oxidative stress *in vivo*. Yet, young $xCT^{-/-}$ mice displayed a deficit in the continuous Y-maze spontaneous alternation task, indicating partial spatial working memory impairment. We observed significantly lower extracellular hippocampal glutamate levels in $xCT^{-/-}$ mice compared to wild type littermates. Moreover, intrahippocampal perfusion with system $x_c^-$ inhibitors lowered extracellular glutamate whereas the system $x_c^-$ activator N-acetylcysteine elevated hippocampal glutamate levels. Correspondingly, xCT deletion in mice elevated the threshold for acute limbic seizures evoked by pilocarpine or kainic acid, and abolished the proconvulsive effects of N-acetylcysteine observed in the kainic acid and the 6Hz corneal stimulation model.

## II Objectives of the project

### II.1 Hypothesis and specific objectives of the project

The above described novel findings thus sustain that system $x_c^-$ is the major source of extracellular glutamate in the hippocampus and striatum, and that genetic deletion of xCT is anticonvulsive in a range of limbic seizure models, does not induce pervasive cognitive effects and results in neuroprotective effects. This breakthrough is the fundament to further unveil the role and mechanisms of action of system $x_c^-$ in (patho)physiological functions.

Moreover, it is now known that activation of synaptic AMPA/NMDA receptors promotes cell survival while the deleterious excitotoxic effects of NMDA receptor activation seem located extrasynaptically, holding lots of promise for extrasynaptic glial targets regulating extracellular glutamate levels, such as the glial system $x_c^-$ on which we focus in the current project.

We hypothesise that upregulation of xCT is involved in the pathological mechanisms of TLE and comorbid major depression; and that deletion or inhibition of system $x_c^-$ will exert neuroprotective or disease modifying effects in these pathological conditions.

The innovative nature of the current proposal is twofold. First, it defines a completely new drug target for epilepsy, and second, this target is localised on glial cells and not on adult nerve cells. As stated in the research priorities for epilepsy, it is important to validate the role of non-conventional mechanisms that control neuronal excitability such as neuron-glia interactions. The study of system $x_c^-$ is in that view an unconventional but relevant approach that can have a major impact on the field of epilepsy and its comorbidities.

Specific research objectives within the frame of the current project proposal as defined by **5 work packages**:

1. **Development of new $x_c^-$ research tools.** As research tools we possess a specific xCT antibody and $xCT^{-/-}$ mice. In the frame of the current proposal we would like to develop mice that overexpress xCT in astrocytes. We also want to contribute to the development of clinically applicable therapeutic strategies that target system $x_c^-$ in epilepsy. Therefore we aim to develop (in collaboration) and test selective non-substrate inhibitors of the cystine/glutamate antiporter.
2. **Immunobiotechnological and molecular biological approaches to study markers of enhanced oxidative stress and/or glutamate excitotoxicity in the brain of $xCT^{-/-}$ mice, xCT overexpressing mice and in rodent models for seizures.** Microarrays as well as real-time PCR will be used to screen a large number of genes related to oxidative stress and antioxidant defence as well as excitotoxicity, glutamate transporters and glutamate receptors, for possible compensatory transcriptional up- or downregulations in hippocampal tissue collected from our $xCT^{-/-}$ mice as well as from epileptic rodents. Interesting data will be confirmed at the protein level with immunohistochemistry and Western blotting techniques.
3. **Neuropharmacological approaches to study transporter properties and functional roles of system $x_c^-$ in the brain of $xCT^{-/-}$ mice, xCT overexpressing mice and in rodent models for seizures.** The applicants have broad experience with in vivo microdialysis, a unique neuropharmacological sampling and delivery tool. Dialysates are analysed for glutamate content or other neuromediators by miniaturised liquid chromatography analysis. In vivo microdialysis will be used in $xCT^{-/-}$ mice, in xCT overexpressing mice as well as during different successive stages of epileptogenesis.
4. **Rodent models for seizures, status epilepticus and epilepsy.** The role of system $x_c^-$ in epilepsy will be investigated in a wide range of validated rodent models of acute seizures and chronic recurrent seizures. We will not only study anticonvulsive mechanisms but will also invest in unveiling a possible true antiepileptic drug target.
5. **Behavioural testing in tasks for memory and antidepressant-like activity.** The role of system $x_c^-$ will also be studied in behavioural tasks for learning and memory in both healthy and epileptic rodents. We indeed aim to develop treatment strategies for the pathophysiological hyperexcitability associated with epilepsy that will not affect the storage mechanisms of long-term memories, or that would improve epilepsy-associated cognitive dysfunction. Possible antidepressant-like effects in mice with deletion or overexpression of xCT will also be studied, because we aim to discover a novel way to treat epilepsy that will not interfere with mood, or that could even ameliorate epilepsy-associated depression.

## III Results obtained in 2012

In 2011, we made progress in specific items described in work packages 3, 4 and 5, as was explained in the GSKE report of last year. These data were compiled into one manuscript that has been published in the Journal of Neuroscience.
In 2012, we made substantial progress in work package 1, 2 and 4; and contributed to a review in the journal Antioxidants & Redox Signaling**.**
*Note: We also finished a last study in the frame of the previous project supported by GSKE.*

### 1 Investigating whether xCT protein expression is altered in human tissue from TLE patients

xCT protein expression levels were compared between human hippocampus of epileptic patients undergoing hippocampal resection for medically intractable TLE and control hippocampal tissue obtained at autopsy from age-matched patients without a history of seizures or other neurologic diseases. These tissues were obtained from the brainbank of the Academic Medical Center (AMC, University of Amsterdam) and the VU University Medical Center (VUMC). Western blotting experiments showed that xCT was significantly upregulated in hippocampus of TLE patients compared to control hippocampus (Figure 3).

### 2 Mechanistic insight into possible intracellular signalling pathways upregulating xCT in TLE

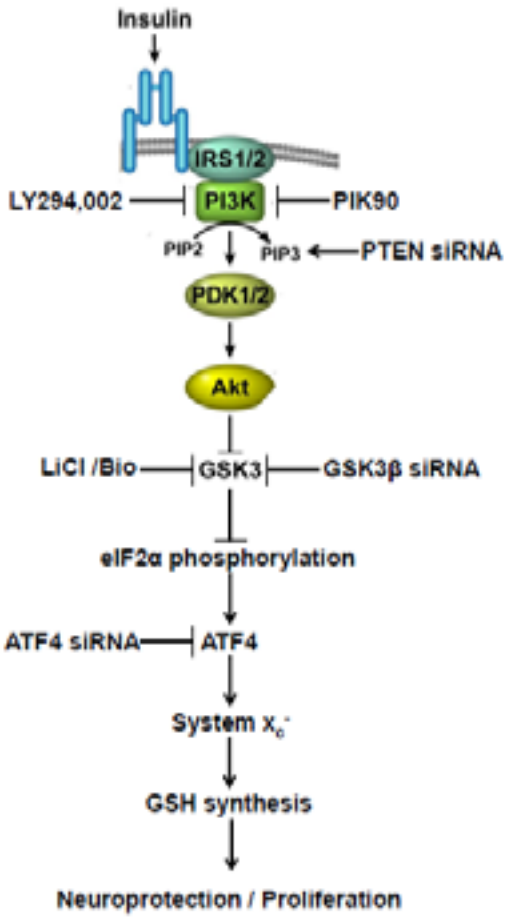


Figure 2 – The PI3K/GSK3β/eIF2α/ATF4/xCT signalling pathway

Initiation of translation usually involves the interaction of certain key proteins with a special tag bound to the 5'-end of the mRNA, the 5' cap. The translation initiation factor eIF2α is part of a multimeric complex that regulates such cap-dependent translation. Phosphorylation of eIF2α (phospho-eIF2α) is induced by various forms of cell stress, resulting in changes to the proteome of the cell.
We contributed to an experimental manuscript (Lewerenz et al., submitted) describing the discovery that phosphorylation of the eIF2α and subsequent upregulation of the transcription factor ATF4 and xCT protein levels, is upstream connected to phosphoinositide3kinase (PI3K) (Figure 2).
This new signalling pathway was also upregulated in the human hippocampal tissue samples of TLE patients.
Indeed, Western blotting performed on five samples of hippocampal tissue from patients with intractable TLE treated with temporal lobectomy compared to five control samples revealed a strong increase in Akt and GSK3β phosphorylation in epileptic tissue which was associated with a significant increase in eIF2α phosphorylation, ATF4 and xCT protein expression (Figure 3).

To support the view that the observed differences between the groups actually represent the activity of the PI3K/GSK3β/eIF2α/ATF4/xCT pathway in the human brain, we performed a linear regression analysis testing the pairwise relationship of the four pairs of connected parts of the pathway across the whole group of samples (Figure 3). This showed positive results for all pairs. Moreover, the linear association of GSK3β and eIF2α phosphorylation showed a goodness of fit (r2=0.72) comparable to those of well-established signalling modules Akt and GSK phosphorylation (r2=0.77) and eIF2α phosphorylation and ATF4 protein expression (r2=0.69) (Lewerenz and Mahrer, 2009).

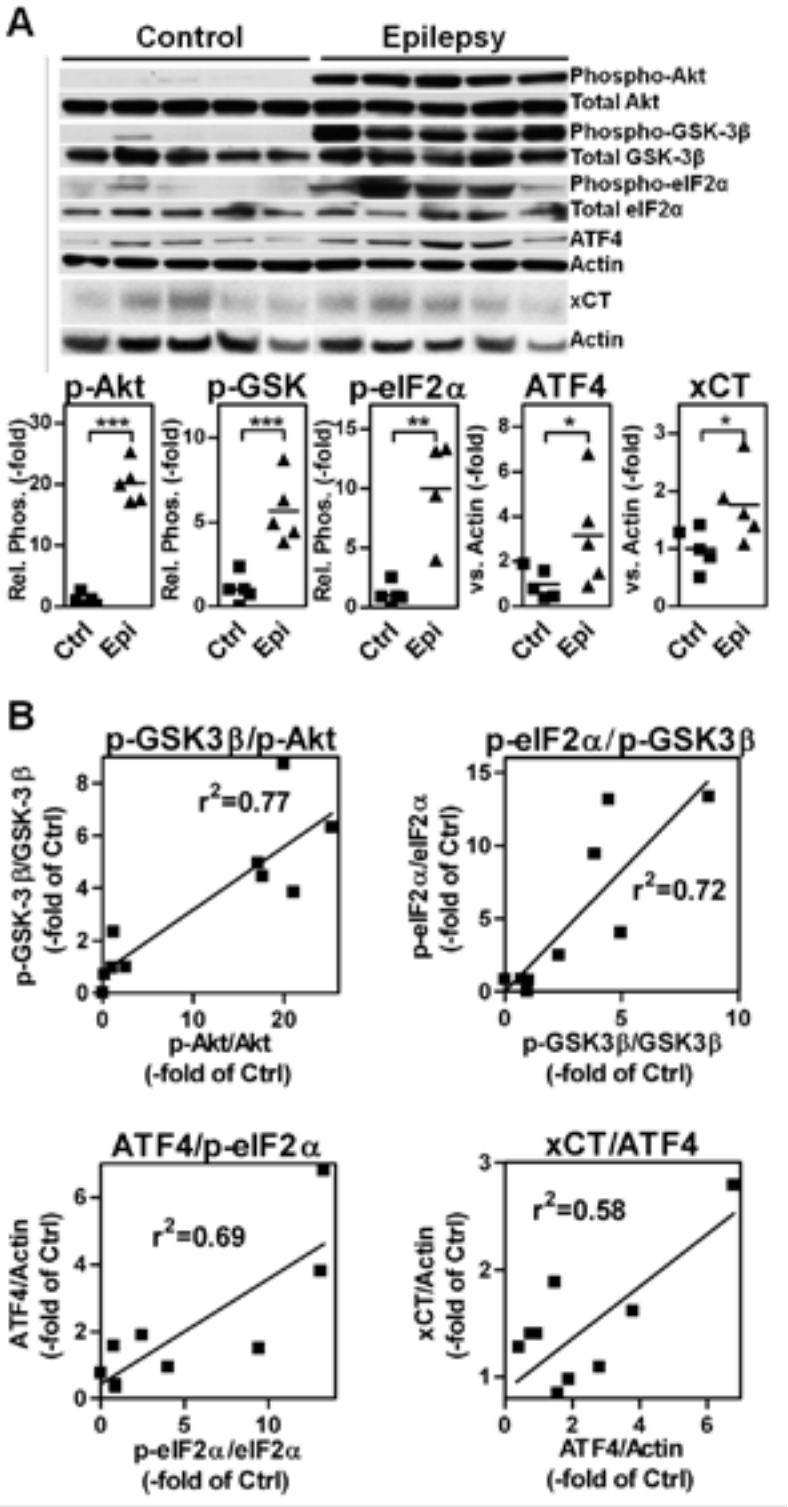


*Figure 3 - The PI3K/GSK3β/eIF2α/ATF4/xCT pathway is activated in human epileptic hippocampal tissue. Protein extracts from surgical specimens from human epileptic hippocampi (Epilepsy) and control hippocampi (Control) were tested for the relative phosphorylation of Akt (phospho-Akt), GSK3β (phospho-GSK3β) and eIF2α (phospho-eIF2α) as well as ATF4 and xCT expression. Either antibodies that recognize the proteins irrespective of their phosphorylation state (total) or actin were used as loading controls. (A) Representative blots are shown. Graphs show the quantitative results with epileptic (Epi) compared to control (Ctrl) tissue with the mean value of the control group normalized to 1. Longer exposures than those shown were used for the quantification of the control samples. For eIF2α phosphorylation, one sample (Epi sample 2, p-eIF2α/eIF2α = 55.8) was excluded as this value classified as an extreme outlier (see Methods). Statistical analysis was performed by one-tailed Student's t tests, ***p<0.001, **p<0.01, *p>0.05. (B) Pair-wise linear regression of the four pairs of connected parts of the pathway across the whole group of samples (Epi sample 2 excluded). The goodness of fit is given in the graphs. The slope was significantly different from zero in all cases (GSK3β/p-Akt, p<0.001; p-eIF2α/p-GSK3β and ATF4/p-eIF2, p<0.01; xCT/ATF4, p<0.05).*

## 3 Study of markers of enhanced oxidative stress and/or glutamate excitotoxicity in the brains of xCT$^{-/-}$ mice

A microarray screening was performed on hippocampal mRNA samples of xCT$^{-/-}$ mice and wild type littermates, in order to identify possible compensatory changes in the xCT$^{-/-}$ mice as a result of the genetic knockout of xCT. We noticed no changes in genes related to oxidative stress or glutamate excitotoxicity. Only two genes were significantly changed between both genotypes and one of these genes may be related to autophagy. Moreover, the phosphoinositide3kinase (PI3K) complex is also involved in autophagy. Knowing that autophagy is recently being linked to many neurological disorders, we will now further investigate whether there is a link between autophagy and system $x_c^-$ and if this link might explain the protective effects we observe in our models for TLE.

## 4 The role of system $x_c^-$ in validated rodent models of epileptogenesis

The models we are currently using for mimicking TLE in mice are the amygdala kindling model, the 6Hz corneal kindling model and the post-status epilepticus pilocarpine model. All are well-accepted models for epileptogenesis, reliably inducing a persistent epileptic state, demonstrable by behavioural and/or EEG assessment. We are currently studying xCT mRNA and protein expression levels and xCT distribution patterns during the various phases of these chronic pilocarpine models for TLE, using C57BL/6 mice. We will also subject xCT$^{-/-}$ and xCT$^{+/+}$ mice (C57BL/6 background) to the various clinically relevant chronic mouse models and compare whether deletion of xCT will affect the process and/or severity of epileptogenesis.

Preliminary data of the 6Hz kindling model using our xCT$^{-/-}$ mice and their wildtype littermates are shown in Figure 4. In this model the process of epileptogenesis is mimicked by twice daily initial subconvulsive corneal stimulations (6Hz, 40mA, 3s) until the mice reach the 'fully kindled state', i.e. 10 consecutive generalized seizures. We noticed a tendency that xCT$^{+/+}$ mice show more severe generalized seizures during this kindling process compared to xCT$^{-/-}$ mice (Figure 4B). Subsequently we try to maintain the 'fully kindled state' by corneal stimulations twice a week. During this maintenance phase we also observed a trend of more generalized seizures in the xCT$^{+/+}$ mice (Figure 4C). These results are suggesting a somehow better outcome of xCT$^{-/-}$ mice, compared to their wildtype littermates, in the 6Hz corneal kindling model.

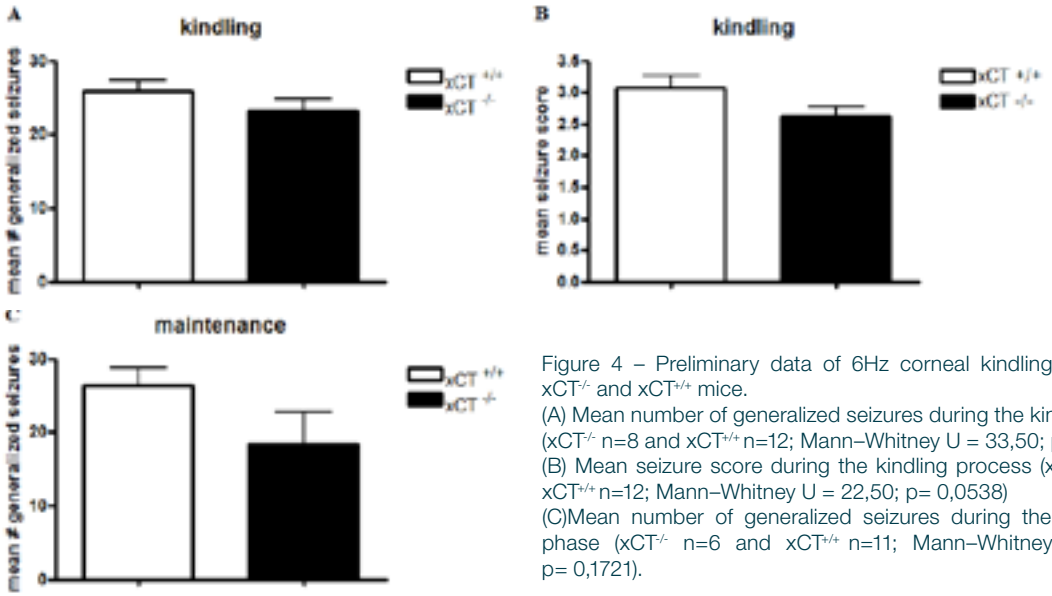


Figure 4 – Preliminary data of 6Hz corneal kindling model using xCT$^{-/-}$ and xCT$^{+/+}$ mice.
(A) Mean number of generalized seizures during the kindling process (xCT$^{-/-}$ n=8 and xCT$^{+/+}$ n=12; Mann–Whitney U = 33,50; p= 0,2796).
(B) Mean seizure score during the kindling process (xCT$^{-/-}$ n=8 and xCT$^{+/+}$ n=12; Mann–Whitney U = 22,50; p= 0,0538)
(C)Mean number of generalized seizures during the maintenance phase (xCT$^{-/-}$ n=6 and xCT$^{+/+}$ n=11; Mann–Whitney U = 19,00; p= 0,1721).

## 5 Development of new $x_c^-$ research tools: In search for an xCT antibody that can be used for immunohistochemistry

In our field, it has become common knowledge that all immunohistochemical stainings for xCT are unreliable, as on fixed tissue many other proteins, besides xCT are being recognised by the existing xCT antibodies. We have been screening a batch of 40 new antibodies (obtained from Dr. N.C. Danbolt, Oslo, Norway) and testing many different staining protocols to come to a result that looks promising. This particular technical realisation that we are currently finalising, will be of utmost importance for the whole community of scientists working on system $x_c^-$ (Van Liefferinge et al., in preparation).

# IV 2012 publication list

### In the frame of the current GSKE project (2011-2013)

- J. Lewerenz, S.J. Hewett, Y. Huang, M. Lambros, P.W. Gout, P.W. Kalivas, **A. Massie, I. Smolders**, A. Methner, M. Pergande, S.B. Smith, V. Ganapathy & P. Maher. The Cystine/Glutamate Antiporter System x(c)(-) in Health and Disease: From Molecular Mechanisms to Novel Therapeutic Opportunities. **Antioxid Redox Signal**. in press, doi: 10.1089/ars.2011.4391. Epub 2012 Aug 3 IF (2011) = 8.45
- J. Lewerenz, P. Baxter, R. Berger, P. Albrecht, **J. Van Liefferinge**, A. Westhoff, P. Meakin, J.D. Hayes, E. Aronica, **I. Smolders**, A.C. Ludolph, A. Methner, **A. Massie**, G.E. Hardingham & P. Maher Phosphoinositide 3-kinases upregulate system $x_c^-$ activity and glutathione synthesis via eIF2α and ATF4 – a pathway active in glioblastomas and epilepsy. Submitted to **Neuron**

### In the frame of the previous GSKE project (2008-2010)

- E. Loyens, D. De Bundel, H. Demaegdt, S.Y. Chai, P. Vanderheyden, **Y. Michotte**, P. Gard, **I. Smolders**. Antidepressant-like effects of oxytocin in mice are dependent on the presence of insulin-regulated aminopeptidase. **Int J Neuropsychopharmacol**. 26 (2012) 1-11. IF (2011) = 4.58

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Progress report of the research group of

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# Charcot-Marie-Tooth neuropathies; from genes to protein networks and disease mechanisms

## 1. Research report:

In the GSKE project 2011-2013 we aim to implement innovative molecular approaches to find novel disease causing genes, and develop strategies to study the “not-obvious” Charcot-Marie-Tooth (CMT) genes. To find functional candidate genes, but also to identify peripheral nerve specific molecular pathways, we aim to pinpoint differential protein–protein interaction networks. In the project we also apply novel approaches to model CMT mutations, validate gene function and interaction networks. In this 2012year report we highlight on our most relevant findings.

In 2012 we reviewed the Hereditary Sensory and Autonomic Neuropathies (HSANs) for *Nature Reviews Neurology* (1). This review highlights the key advances in the understanding of HSANs, including insights into the molecular mechanisms of disease, derived from genetic studies of patients with these disorders. The HSANs, also known as ulcero-mutilating neuropathies, are a particular group of rare devastating diseases of the peripheral nervous system characterized by profound sensory loss, acral mutilations and variable autonomic disturbances. Because of severe mutilations, patients will often have amputations of their fingers, toes or even limbs. The clinical picture of HSAN disorders impressively demonstrates the importance of sensory innervations to protect the body from injury. The prevalence of HSAN is estimated at 1/10.000 and the genetic spectrum encompasses autosomal dominant and autosomal recessive forms, with 108 causative mutations identified in 13 genes so far. However, the genetic cause still remains unresolved in at least two-thirds of patients with HSAN. Recently, promising treatment options for a few genetic subtypes have been demonstrated. A better understanding of the underlying pathomechanisms, with the final goal to find therapeutic approaches, will improve the quality of life of patients with inherited but also acquired ulcero-mutilating neuropathies. Research on HSAN will help to elucidate the mechanisms contributing to long-term neuronal survival, which is not only subject of rare diseases, but also relevant for common disorders of the ageing society.

We also reviewed the CMT neuropathies and hereditary spastic paraplegias (HSPs) for *Experimental Neurology* (2). A key difference between these two groups of conditions is in the target cells that they primarily affect, with CMT neuropathies affecting the peripheral motor and sensory nerves, while the HSPs principally affect the central nervous system axons of the corticospinal tract and dorsal columns. However, CMT neuropathies and HSPs also share many features, for example, both are genetically determined long axonopathies that affect motor and sensory pathways and which can be later-onset and are typically progressive. This commonality suggests that there might be similarities in the molecular pathology underlying these conditions, and in this review manuscript we compared and contrast the molecular genetics and cellular pathology of the two groups of neurodegenerative diseases. In this context we recently contributed to an whole exome sequencing (WES) project and identified a REEP1 mutation involved in distal hereditary motor neuropathy type V (distal HMN type V). Our data corroborate the loss-of-function nature of REEP1 mutations in HSP and suggest that a different mechanism applies in REEP1-associated distal HMN type V (3).

We completed in 2012 a study on the use of skin fibroblasts of distal hereditary motor neuropathy (distal HMN type II) patients, and reported that mutant small heat shock protein HSPB8 causes protein aggregates and a reduced mitochondrial membrane potential in dermal fibroblasts from distal HMN

patients (4). To further advance in the direction of treatment for these motor types of CMT neuropathies, we currently aim to develop motor neuron cultures differentiated from patient-derived induced pluripotent stem cells (iPSC), a promising and novel approach in translational research that will allow expansion of our knowledge on disease mechanisms, and development of an efficient and relevant drug testing platform for therapeutic intervention in CMT. In-house developed biochemistry tools and microscopy imaging assays have been developed and will be used on iPSC-derived motor neurons to assess axonal outgrowth, -degeneration and -transport. An important advantage of this strategy is the ability to test the effect of potential therapeutics in a genetically matched, reproducible, and up-scalable human cell model. The results obtained can be expanded to mutations in other CMT associated genes.

In 2012, we elaborated in *BioArchitecture* (5) on two of our most recent studies showing that mutations in the small heat shock protein HSPB1, which cause an axonal type of CMT neuropathy, affect microtubule dynamics and impede axonal transport (6,7). While at presymptomatic age the neurons in the mutant HSPB1 mouse show a hyperstable microtubule network, at postsymptomatic age, the microtubule network completely lost its stability as reflected by a marked decrease in tubulin acetylation levels. We proposed in our review manuscript a model explaining the role of microtubule stabilization and tubulin acetylation in the pathogenesis of HSPB1 mutations (5). In September 2012, one of our PhD students, Anne Holmgren, completed her thesis on the "Molecular biology of small heat shock protein mutations associated with CMT and distal hereditary motor neuropathies". She contributed to the development of a knock-out/knock-in mouse model for HSPB1 (unpublished results), and studied the interaction between HSPB1 and the neurofilaments. Parts of her PhD thesis included reviews on the small heat shock proteins and the neurofilaments in heath and disease (8,9).

Finally, we recently reported the identification of a novel gene for CMT and neuromyotonia in *Nature Genetics* (10). In 33 families, we identified 8 mutations in *HINT1* (encoding histidine triad nucleotide–binding protein 1) by combining linkage analyses with next-generation sequencing and subsequent cohort screening of affected individuals. Our study provided evidence that loss of functional HINT1 protein resulted in a distinct phenotype of autosomal recessive axonal neuropathy with neuromyotonia. The myotonia is characterized by the slow relaxation of muscles following voluntary exertion. Hypersensitivity of the nerve or muscle is likely at the origin of this occurrence. The finding of HINT1 will contribute to the development of better diagnostic tools for peripheral neuropathies and conditions accompanied by myotonia. It also demonstrates that HINT1 protein plays an important role in the functioning of the peripheral nervous system. Unfortunately, it is at present still unclear how mutations in the HINT1 gene cause peripheral neuropathies associated with myotonia.

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# 2. Research Activities in 2012:

## Articles in International Journals – Acknowledging the GSKE:

- De Almeida Souza,L., **Timmerman,V.,** Janssens,S.: Microtubule dynamics in the peripheral nervous system: A matter of balance. BioArchitecture 6: 267-270 (2011) (PMID: 22545178) (this on line paper was not included in our previous GSKE report as the publication was only available early 2012).
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## Scientific Prizes:

- **P. De Jonghe and V. Timmerman:** Medical Foundation Queen Elisabeth, GSKE-Valine De Spoelberch Prize, Brussels, Belgium, May 15, 2012
- **J. Baets:** Koninklijke Academie voor Geneeskunde van België, Prijs voor Klinisch Wetenschappelijk Onderzoek in de Geneeskunde 2012, 'Genotype-phenotype correlations in hereditary neuropathies: a systematic approach', November 17, 2012

## Awards and fellowships:

- **J. Baets:** Peripheral Nerve Society, Arthur K. Asbury Travel Grant, 2012 PNS – INC Congress, Rotterdam, The Netherlands, June 24 – 27, 2012
- **J. Baets:** 2012 FWO travel grant, American Society of Human Genetics meeting, San Francisco, USA.
- **E. Ydens:** Peripheral Nerve Society, Rabobank Travel Grant, 2012 PNS-INC Congress, Rotterdam, The Netherlands, June 24-27, 2012

## PhD theses:

- **A. Holmgren:** "Molecular biology of small heat shock protein mutations associated with Charcot-Marie-Tooth and distal hereditary", Promotor: Timmerman V., Irobi J., September 10th 2012

## Master theses:

- **D. Atkinson:** "Characterization of a *Drosophila* model for Charcot-Marie-Tooth type 2B", Supervisor: K. Janssens, Technician: B. Asselbergh (Academic MSc Thesis, UA)

## Bachelor theses:

- **E. Lefèvere:** "Genetische analyse van REEP1 in distale HMN cohorten", Supervisor: J. Baets, Technician: T. Deconinck (Academic BSc Thesis)
- **W. Benoey:** "Moleculair en celbiologisch onderzoek van de ziekte van Charcot-Marie-Tooth", Supervisor: V. Timmerman, A. Holmgren, Technician: V. De Winter (Academic BSc Thesis)

## Chair and organizational activities

- **V. Timmerman and P. De Jonghe:** Inherited Neuropathies Consortium (INC), Rare Disease Clinical Research Consortium (RDCRC), EAB meeting in Miami, USA, February 15-17, 2012, Member of the External Advisory Board

## Invited Lectures:

- **P. De Jonghe:** "Hereditary Neuropathies, an overview". 4. Neuromuskuläres Symposium, Neuropathien bei Kindern und Erwachsenen', Zürich, November 22, 2012 (Plenary Lecture)
- **V. Timmerman:** "Understanding the pathomechanisms of Charcot-Marie-Tooth neuropathies", Thomas Wahlig Foundation, Köln, Germany, March 16, 2012 (Plenary Lecture)
- **V. Timmerman:** "Understanding the pathomechanisms of inherited peripheral neuropathies", European Society of Human Genetics (ESHG) meeting 2012, Nürnberg, Germany, June 24-26, 2012 (Educational Lecture)

## Slide presentations selected at international meetings:

- **J. Baets:** "Autosomal Recessive Axonal Neuropathy with Neuro-Myotonia: a novel disease entity caused by mutations in HINT1", American Society of Human Genetics, San Francisco, USA, November 6-10, 2012
- **J. Baets:** "Autosomal recessive axonal neuropathy with neuromyotonia: a new disease entity", Peripheral Nerve Society satellite meeting, Rotterdam, The Netherlands, June 24-27, 2012
- **E. Ydens:** "Macrophage activation in Wallerian degeneration", Peripheral Nerve Society – Inflammatory Neuropathy Consortium (PNS-INC), Rotterdam, The Netherlands, June 24-27, 2012

## Poster presentations at international meetings:

- **B. Asselbergh:** "Peripheral neuropathy mutants stabilize microtubules and reveal a novel role for HSPB1 in microtubule nucleation", Novel Biophysical Approaches in the Investigation of the Cytoskeleton, the 27th European Cytoskeleton Forum Meeting, Pécs, Hungary, November 3-7, 2012
- **F.M. Ipek:** "Modelling a sensory neuropathy caused by mutations in SPTLC2 in Drosophila", GRC: Glycolipid and sphingolipid biology, Lucca, Italy, April 22-27, 2012
- **F.M. Ipek:** "Modelling a sensory neuropathy caused by mutations in SPTLC2 in Drosophila", Neurofly, Padova, Italy, September 3-7, 2012
- **K. Janssens:** "Modelling CMT2B, caused by mutations in RAB7A, in Drosophila melanogaster", Rab GTPases and their interactors in health and disease, Cork, Ireland, June 11-13, 2012
- **E. Ydens:** "Macrophage activation in Wallerian degeneration", Peripheral Nerve Society – Inflammatory Neuropathy Consortium (PNS-INC), Rotterdam, The Netherlands, June 24-27, 2012

## Invited lectures at national meetings

- **V. Timmerman**: "Recente resultaten uit het CMT onderzoek en mogelijkheden voor de toekomst", VSN congres, Veldhoven, Nederland, September 15, 2012
- **V. Timmerman:** "Understanding the pathomechanisms of inherited peripheral neuropathies", GIGA Neuroscience –Ulg, Luik, November 6, 2012

## Slide presentations selected at national meetings:

- **L. Almeida-Souza:** "Hyperactivity and microtubule network stabilization underlies the pathogenesis of HSPB1 mutations in Charcot-Marie-Tooth neuropathy", VIB Seminar 2012, Blankenberge, Belgium, April 18-20, 2012
- **I. Mademan:** "Whole Exome Sequencing in CMT: waarom en is dit wel nuttig?" CMT jaarlijkse studie- en contactdag, Wilrijk, Belgium, October 27, 2012
- **L. Peeraer:** "Geïnduceerde pluripotente stamcellen voor CMT, waarom en is dit wel nuttig?" CMT studie- en contactdag, Antwerp, Belgium, October 27, 2012
- **E. Ydens:** "Acute neurodegeneration in the peripheral nervous system triggers an alternative macrophage response", VIB Seminar 2012, Blankenberge, Belgium, April 18-20, 2012

## Poster presentations at national meetings:

- **B. Asselbergh:** "An automated image analysis procedure to measure neurite outgrowth on phase contrast images of neurons in culture", 12th International meeting on advanced light microscopy, Leuven, Belgium, June 5-8, 2012

Progress report of the research group of

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# Celsr genes in brain development and function

## Work progress

During 2012, we have mainly focused on the role of Celsr1-3 in ependymal cilia polarity. In the mouse forebrain, ependymal cells lining cerebral ventricles bear at their apical surface multiple cilia that beat in concerted manner to assist the circulation of the cerebrospinal fluid (CSF) (Ibanez-Tallon, Pagenstecher et al. 2004; Lechtreck, Delmotte et al. 2008; Tissir, Qu et al. 2010). The base of each cilium displays a polarized organization best evidenced by the presence of asymmetric appendages extending from basal bodies (BB) particularly the basal foot, an arrowhead-like structure that points towards the active stroke of cilia beat. In differentiating ependymal cells, cilia appear randomly oriented (Guirao, Meunier et al. 2010). They subsequently rotate and progressively assume a common orientation, a process termed rotational polarity (Guirao, Meunier et al. 2010; Mirzadeh, Han et al. 2010). This polarity is also seen across the tissue in that cilia from adjacent cells point to the same direction. Furthermore, BBs that are initially widely scattered regroup into an off-centered patch at the anterior tier of the apical surface, a feature referred to as translational polarity (Mirzadeh, Han et al. 2010). Ependymal cells differentiate perinatally from radial glia (RG) progenitors which carry a single primary cilium (Merkle, Tramontin et al. 2004). Like motile multicilia, the primary cilium is displaced from the centre of the apical domain, and this anterior shift, shared by neighboring cells, is the first sign of tissue polarity.

We first wondered whether PCP affects the polarity of the primary cilium and tested this hypothesis using the following mutants: *Celsr1*$^{-/-}$, Celsr2$^{-/-}$ *Celsr2*$^{Dgen/Dgen}$, *Celsr3*$^{-/-}$, *Fzd3*$^{-/-}$ and *Vangl2*$^{cKO}$. We analyzed “en face” preparations of newborn (P0–P1) lateral wall (LW), with emphasis on its anterior part to minimize regional variations. We found that, like in the wildtype (WT), RG cells had a primary cilium in all PCP mutants. We evaluated the relative distance between the centre of the apical cell surface and the BB and found that the primary cilium was displaced from the cell centre in all genotypes (WT=0.41±0.02; *Celsr1*$^{-/-}$=0.42±0.03, p=0.4101; *Celsr2*$^{-/-}$=0.43±0.02, p=0.1467; *Celsr2*$^{Dgen/Dgen}$=0.44±0.04, p=0.1208; *Celsr3*$^{-/-}$=0.44±0.02, p=0.0794; *Fzd3*$^{-/-}$=0.40±0.04, p=0.6857; *Vangl2*$^{cKO}$=0.43±0.02, p=0.2618). We analyzed the direction of BB displacement by drawing a vector from the cell center to the BB. Vectors from neighboring cells pointed to similar directions in WT, *Celsr2*$^{-/-}$, and *Celsr3*$^{-/-}$. In contrast, they were randomized in *Celsr1*$^{-/-}$, *Celsr2*$^{Dgen/Dgen}$, *Fzd3*$^{-/-}$, and *Vangl2*$^{cKO}$. Analysis by circular statistics showed that the angular deviation of individual vectors from the mean was comprised between -45° and +45° in WT, *Celsr2*$^{-/-}$, and *Celsr3*$^{-/-}$ samples, but displayed broader distributions in *Celsr1*$^{-/-}$, *Celsr2*$^{Dgen/Dgen}$, *Fzd3*$^{-/-}$, and *Vangl2*$^{cKO}$ mutants. These results show that PCP genes *Celsr1*, *Fzd3* and *Vangl2* coordinate the positioning of the primary cilium in radial progenitors.

We have already shown that loss of function of Celsr2 and Celsr3 impairs rotational polarity of ependymal cilia and results in defective flow of CSF and lethal hydrocephalus (Tissir, Qu et al. 2010). Celsr2&3 mutant ependymal cilia never develop in normal numbers and display abnormalities in morphology, position, and planar organization. Ciliary basal feet are disoriented, and basal bodies were seen ectopically deep in the cytoplasm. The lateral plasma membrane localization of Vangl2 and Frizzled3 is disrupted in ependymal cells, indicating that Celsr2 and Celsr3 act via PCP to regulate the docking of basal bodies and the apical positioning of cilia. Rotational polarity is usually assessed by analyzing the orientation of the basal foot by transmission electron microscopy. This method is tedious and time consuming. To speed up the study of ependymal polarity in other PCP mutants, we sought to develop an alternative to electron microscopy. We test many markers and found that gamma tubulin and phosphorylated

beta catenin localize at distinct positions in the BB and define a vector “Vcil” that can used to evaluate cilia polarity. Using Immunofluorescence, we compared the organization of ciliary basal body (BB) patches between controls and the following mutants: *Celsr1*$^{-/-}$, *Celsr2*$^{Dgen/Dgen}$, and *Celsr2*$^{-/-}$. We also generated forebrain conditional knockouts (*Celsr3*$^{cKO}$ and *Vangl2*$^{cKO}$) by crossing *Celsr3*$^{+/-}$;FoxG1-*Cre* and *Vangl2*$^{+/-}$;FoxG1-*Cre* males with Celsr3$^{f/f}$ and Vangl2$^{f/f}$ females. In the wild type P21, ciliary patches exhibited a stereotypic organization: BBs were aligned in parallel rows, with roughly the same number of BBs per row and a regular spacing. Furthermore, they were uniformly oriented and accordingly, low circular standard deviations (CSD) of Vcil were obtained. We defined the mean of Vcil as the vector of patch orientation “VpatchO”, an indicator of rotational polarity. In *Celsr1*-deficient cells, the stereotypic arrangement was relatively preserved. In contrast, it was markedly impaired in *Celsr2*$^{-/-}$, *Celsr3*$^{cKO}$ and *Vangl2*$^{cKO}$ mutant cells. In *Celsr2*$^{-/-}$cells, whereas the orientation of cilia in a given patch appeared coordinated, the number and spacing of BBs varied from one row to the other, thus affecting the shape of the patch. In *Celsr3*$^{cKO}$ and *Vangl2*$^{cKO}$cells, cilia failed to adopt a uniform alignment in the patch, and sometimes displayed oblique or even perpendicular rows, resulting in higher CSDs than in WT. Remarkably, a global orientation of Vcil, and thus VpatchO could be defined in cells with a CSD below 40°. To estimate the frequency of these cells, we calculated the Vcil CSD for more than 300 cells per genotype and ranked their distribution in different bins. In WT, the CSD was below 20° in 83.5% of cells, between 20 and 40° in 16.5% cells, and above 40° in a negligible fraction (0.29%). All mutants displayed a trend towards higher CSD than in WT, reflecting a relative disorganization of patches. Nevertheless, in all mutants, the proportion of cells with a CSD inferior to 40° was above 80% (*Celsr1*$^{-/-}$: 91.2%, *Celsr2*$^{-/-}$: 99.6%, *Celsr3*$^{cKO}$: 84.2%, *Vangl2*$^{cKO}$: 86%). These results show that at the single cell level, Celsr2, Celsr3 and Vangl2 control the intrinsic organization of BB patches.

Another feature of ependymal cilia polarity is that BB patches are displaced from the center of the apical domain and display a preferential orientation. We then asked whether PCP is involved in this processes at the tissue level. We therefore studied cilia development and patch formation and positioning in *Celsr1*$^{-/-}$, *Celsr2*$^{-/-}$, *Celsr3*$^{flox/-}$; *FoxG1-Cre* (*Celsr3*$^{cKO}$), and *Vangl2*$^{cKO}$ mutants. Like in WT, BB patches were displaced away from the center of the apical surface in PCP mutants. We evaluated the magnitude of patch displacement by measuring the relative distance between the center of the apical surface and that of the patch, and found no difference between WT, *Celsr1*$^{-/-}$, and *Celsr3*$^{cKO}$ cells (WT: 0.40±0.02, 1193 cells from 5 animals; *Celsr1*$^{-/-}$: 0.38±0.03, p=0.1859, 1107 cells from 5 animals; Celsr3$^{cKO}$: 0.38±0.02, p=0.1764, 439 cells from 3 animals). A slight reduction was observed in *Vangl2* and *Celsr2* mutant cells (*Vangl2*$^{cKO}$: 0.35±0.02, p=0.0086, 1013 cells; *Celsr2*$^{-/-}$: 0.34±0.01, p= 0.0007, 730 cells from 5 animals per genotype). However, this difference reflected the fact that BB patches remained at the center of the apical surface in some cells in *Vangl2*$^{cKO}$ mice and exhibited an abnormal shape in *Celsr2*$^{-/-}$ mice, rather than a decreased magnitude of displacement. These results demonstrate that, in the absence of PCP proteins, ependymal cells remain able to cluster their BBs in an off-centered patch.

In individual mutant cells, BB patches are displaced from the center of the apical domain and display a preferential orientation. We then asked whether PCP coordinates these processes at the tissue level. To address this, we defined a vector “VpatchD” from the center of the apical surface to the center of the patch. This vector reflects the direction of patch displacement and is an indicator of translational. We used VpatchD and VpatchO to compare respectively the direction of displacement and the orientation of ciliary patches in large fields of the mature ependyma. In WT, the direction of patch displacement was similar in neighboring cells as reflected by comparable VpatchDs. Likewise, the patch orientation was uniform with VpatchOs systematically pointing to similar directions. In contrast, in *Celsr1*$^{-/-}$ and *Vangl2*$^{cKO}$ mutants, we observed a randomization of patch displacement and orientation: VpatchDs of adjacent

cells sometimes pointed to, or opposed each other, and VpatchOs pointed in different directions. We quantified this by measuring angles between individual VpatchDs, VpatchOs and their respective means. These angles were comprised between -45° and +45° in WT, and displayed a broader circular distribution in *Celsr1*$^{-/-}$ and *Vangl2*$^{cKO}$ mutants, confirming the dispersal of patch displacement and orientation. Analysis of *Celsr2*$^{-/-}$ and *Celsr3*$^{cKO}$ mutants did not reveal any striking difference in direction of patch displacement, or orientation. These results demonstrate that Celsr1 and Vangl2 are required for the intercellular coordination of patch displacement and patch orientation.

In WT cells, the direction of patch displacement coincides with patch orientation. We asked whether this is preserved in PCP mutants. In WT cells, VpatchD and VpatchO indeed pointed to the same direction. This correlation was lost in *Celsr1* mutants, where we frequently observed cells with perfectly organized ciliary patches, yet a VpatchO pointing in the opposite direction to VpatchD. A similar phenotype was observed in *Vangl2*$^{cKO}$, but not in *Celsr2*$^{-/-}$ or *Celsr3*$^{-/-}$ cells. We quantified this by measuring angles between VpatchD and VpatchO. Whereas this angle was inferior to 45° in WT, *Celsr2*$^{-/-}$, and *Celsr3*$^{cKO}$, large angles, up to 180°, were observed in *Celsr1* and *Vangl2* mutant samples. These results demonstrate an uncoupling between the direction of patch displacement and patch orientation in absence of functional Celsr1 and Vangl2.

## In summary:

- We studied ependymal polarity in PCP mutant lines, namely: *Celsr1*$^{-/-}$, *Celsr2*$^{Dgen/Dgen}$, *Celsr2*$^{-/-}$ (a new mutant generated in this study), *Fzd3*$^{-/-}$, *Vangl2*$^{f/-}$;*FoxG1-Cre*, and *Celsr3*$^{f/-}$;*FoxG1-Cre*.
- We developed a novel immunostaining-based method as an alternative to electron microscopy. This method reliably shows the orientation of individual cilia and facilitates the analysis of individual cells as well as large fields with dozens of cells.
- We defined and used rigorous and new parameters that outline the orientation of individual cilia, the overall orientation of cilia in a given cell, and their positioning relative to the centre of the cell. These parameters provide an accurate and exhaustive way to assess rotational and translational polarities.

Using these novel genetic and cellular tools:
- We show for the first time that PCP controls polarity of the primary cilium in radial glial progenitors through Celsr1, Fzd3 and Vangl2.
- In ependymal cells, we identify an as yet unreported function of PCP in translational polarity.
- We show that two PCP signals, sorted by different Celsrs, act concomitantly to organize different levels of polarity. The first signal involves Celsr2&3 and Vangl2 and controls the intracellular organisation of cilia. The second implicates Celsr1 and Vangl2 and coordinates different aspects of planar polarity at the single cell and tissue levels. These two signals trigger distinct changes in the cytoskeleton.
- Our results lead us to propose an integrated view of how planar polarity is established and propagated in time and space during the transition from radial glia to ependymal cells.

These results were compiled in a manuscript that has been recently submitted for publication.

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Progress report of the research group of

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# Study of the role of the NF-κB regulatory protein A20/TNFAIP3 in central nervous system inflammation

Multiple sclerosis (MS) is the most common chronic inflammatory demyelinating disease of the central nervous system (CNS). The cause of degeneration in MS remains largely enigmatic, but is generally considered to result from an autoimmune inflammatory reaction leading to demyelination and axonal damage in the CNS. The disease is characterized by activated auto-reactive myelin-specific lymphocytes which home to the CNS, where they initiate a vicious cycle of inflammation and tissue damage. The major cellular targets in MS pathology are oligodendrocytes, the myelin producing cells of the CNS, and neurons. Their loss is directly associated with clinical manifestations of the disease, including sensation deficits, optic neuritis and progressive paralysis. Much knowledge about MS has resulted from studies in rodents subjected to experimental autoimmune encephalomyelitis (EAE), the main animal model of MS. Studies using gene-targeted deficient and transgenic mice have established the role of the peripheral immune system and of multiple chemokines and cytokines in EAE pathology. Proinflammatory cytokines and chemokines are produced by both infiltrating immune cells and resident CNS glial cells, orchestrating a pathogenic cascade leading to inflammation, demyelination and axonal damage.

Inflammatory responses are regulated by intracellular signalling pathways initiated by the activation of innate immune receptors and cytokine receptors. A crucial transcription factor controlling inflammatory responses is NF-κB. Many different stimuli result in NF-κB activation, through the activation of the IκB kinase (IKK), leading to the expression of pro-inflammatory genes. NF-κB activation in peripheral immune cells is absolutely essential for the induction of EAE pathology, and although little is still known about the involvement of NF-κB in inflammation locally in the CNS, we could recently show a brain-specific role for IKK-dependent NF-κB activation in the pathology of EAE (van Loo *et al.,* 2006; van Loo *et al*., 2010). Similar observations were done in a second mouse model for CNS demyelination induced by the neurotoxicant cuprizone (Raasch, van Loo *et al*., 2011).

## 1 A20/TNFAIP3 in central nervous system inflammation

As NF-κB activation is so crucial in many biological cellular processes, it is not surprising that a tight regulation of the pathway and the genes induced is an absolute requirement. For this, cells employ a multilayered control system to keep immunity and inflammation in check, and the combined action of different positive and negative regulators help to fine-tune the immune response. One critical brake on NF-κB activation is A20/TNFAIP3 (TNFα induced protein 3). A20 is a cytoplasmic zinc finger protein that has been characterized as a dual inhibitor of NF-κB activation and apoptosis (Vereecke *et al*., 2009). In most cell types, A20 expression is very low without stimulation but is rapidly transcriptionally induced by NF-κB. Once expressed, A20 functions as a negative feedback regulator of NF-κB activation. The essential role of A20 in the regulation of NF-κB and apoptotic signalling was demonstrated through the generation of a complete A20 knockout mouse (Lee *et al*., 2000). Mice deficient for A20 develop severe inflammation and cachexia, are hypersensitive to LPS and TNF, and die prematurely. A20-deficient cells fail to terminate TNF-induced NF-κB responses and are more susceptible to TNF-mediated apoptosis. Besides its critical role for the regulation of TNF-receptor-dependent pro-inflammatory signals, A20 is also required for termination of Toll-like receptor (TLR) and Nucleotide-binding Oligomerization Domain

containing 2 (NOD2) receptor responses. Interestingly, *A20/TNFAIP3* has been identified in humans as a susceptibility locus for multiple immunopathologies including Crohn's disease, systemic lupus erythematosus and rheumatoid arthritis (RA) (reviewed by Vereecke *et al.,* 2009). Importantly, we could recently confirm these associations using mice with a conditional A20 knockout allele, allowing tissue-specific A20 deletion (Vereecke *et al*., 2010; Kool *et al.,* 2011; Matmati *et al*., 2011). These findings clearly indicate a crucial and cell type specific role for A20 in controlling inflammatory immune responses. Interestingly, genome-wide association studies also suggested *A20/TNFAIP3* as a susceptibility gene for multiple sclerosis (De Jager *et al*., 2009; Gilli *et al.,* 2011).

One of the aims of our GSKE-funded project is to understand the function, activation and regulation of A20 in the development and progression of central nervous system inflammation and demyelination. The basis approach is to genetically manipulate the A20 gene in mice in specific neuronal populations and immune effectors and to determine the effects of such mutation in development and inflammatory disease pathogenesis.

### 1.1 A20/TNFAIP3 in MS/EAE pathology

To study the CNS-specific role of A20 in the immunopathology of MS, we make use of the experimental MS model EAE, which can be induced by immunization of mice with myelin oligodendrocyte glycoprotein (MOG) or other encephalogenic agents. To evaluate the cell specific contribution of A20 in the pathogenesis of EAE, different tissue-specific A20 knockout mice (all-CNS-, neuron-, astrocyte-, oligodendrocyte-specific, T cell-, myeloid cell- and DC-specific) were generated from a conditional 'floxed' A20 knockout mouse (Vereecke *et al*., 2010), and subjected to MOG-peptide-induced EAE. The clinical course for disease initiation and progression is followed and spinal cord sections are evaluated for inflammatory infiltrates and demyelination. Furthermore, inflammatory cytokine and chemokine production is measured by quantitative real time PCR. The capacity of macrophages and DCs in T cell activation is further analysed *in vitro* using primary cultures isolated from respective A20 knockout mice and control littermates. Similarly, primary neuronal cultures are used *in vitro* to establish the impact of A20 deficiency on inflammatory challenge. Next to the EAE model of MS, brain-specific demyelination can also be induced by putting mice on a diet containing the neurotoxicant cuprizone. In this model, administering cuprizone for 6 weeks causes complete demyelination of the corpus callosum in the absence of an immune reaction. Furthermore, when this administration is terminated, complete remyelination of the corpus callosum occurs, rendering this model useful to study both de- and remyelination. CNS demyelination, and astro- and microgliosis are evaluated by histology on corpus callosum sections, and inflammatory cytokine and chemokine expression by qPCR (Mc Guire *et al*., ongoing studies).

### *1.2 A20/TNFAIP3 in cerebral ischemia*

Cerebral ischemia is characterized by the activation of glial cells, causing a rapid and massive local inflammatory reaction leading to tissue damage and neuronal cell death (Ridder and Schwaninger, 2009). Over the past decade, it has become increasingly clear that NF-κB plays a central role in the pathogenesis of cerebral ischemia. NF-κB gets activated in the ischemic hemisphere shortly after permanent middle cerebral artery occlusion (MCAO). Furthermore, NF-κB deficient mice show a reduction in ischemic damage after transient or permanent MCAO, suggesting a cell death promoting role for NF-κB in this model. In line with these, mice lacking IKK2 in all neuroectodermal cells or specifically in neurons show a decreased infarct volume 48 hours after pMCAO, whereas constitutive activation of IKK2 increased infarct size (Herrmann *et al.,* 2005). However, NF-κB may also act beneficial in conditions of ischemic preconditioning which was shown to protect against a subsequent prolonged ischemic insult through transcriptional activition of NF-κB.

Since A20 is a key negative regulator of NF-κB signaling, but can also act as a strong anti-apoptotic protein, we sought to clarify the *in vivo* role of A20 in the MCAO model of brain ischemia in mice. We demonstrated that NF-κB driven genes such as *A20, TNF* and *IL-6* are upregulated in the infracted area 24 hours post pMCAO. In agreement with these *in vivo* data, A20 mRNA was also upregulated in primary murine cortical neurons when placed in a glucose deprived 0,1% $O_2$ hypoxic environment for four hours *in vitro*. Since A20 seems to be differentially regulated both *in vivo* and *in vitro* during ischemic conditions, we questioned whether mice lacking A20 specifically in the CNS ($A20^{CNS\text{-}KO}$) or exclusively in neurons ($A20^{NEUR\text{-}KO}$) would be affected differently after pMCAO when compared to wild type littermates. $A20^{CNS\text{-}KO}$ or $A20^{NEUR\text{-}KO}$ mice, together with control littermate mice, were subjected to pMCAO for 24 hours after which the infarct size was estimated by means of a silver staining technique. To our surprise the infarct volume after 24 hours of pMCAO did not differ between $A20^{CNS\text{-}KO}$ or $A20^{NEUR\text{-}KO}$ and their respective wild type control littermates. Collectively, these results clearly demonstrate that, although A20 is upregulated in conditions of pMCAO, the lack of A20 in either all cells of neuroectodermal origin or neurons in particular does not influence the outcome of pMCAO in mice (Mc Guire *et al*., submitted for publication).

## 2 Mucosa-associated lymphoid tissue 1 (MALT1) in MS/EAE

NF-κB plays a central role in the activation and proliferation of T cells. Upon stimulation of the T cell receptor (TCR), protein kinase C (PKC) θ-mediated phosphorylation of the caspase activation and recruitment domain (CARD)-containing protein CARMA1 (also known as CARD11) results in the recruitment of B cell lymphoma-10 (BCL10) and mucosa-associated lymphoid tissue 1 (MALT1) (Staal *et al*., 2011). This CARMA1/BCL-10/MALT1 (CBM) complex subsequently recruits TNF receptor associated factor (TRAF) 2 and 6 allowing further downstream signaling, leading to nuclear translocation and activation of NF-κB. Gene targeting strategies have shown that MALT1 is indispensable for NF-κB activation downstream of TCR stimulation, resulting in an absence of T cell activation and proliferation in MALT1 deficient T cells. Besides acting as a scaffold mediating TCR signaling, MALT1 also has proteolytic activity. Indeed, recent findings have demonstrated that A20, Bcl10, RelB and CYLD are substrates of MALT1. Although the adaptor function of MALT1 is indispensable for T cell activation, its proteolytic activity is considered to be critical for a full-blown NF-κB response, shaping the extent of T cell activation (Staal *et al*., 2011).

Because of its essential role in T and B cell activation, MALT1 is considered an important therapeutic target in autoimmunity. However, so far its role in the development of autoimmune disease has not been reported. We sought to address the role of MALT1 in the generation of autoreactive T cells in the context of EAE. For this, we induced EAE in mice deficient in MALT1 ($MALT1^{-/-}$) and in wild-type and heterozygous littermate control mice ($MALT1^{+/+}$ and $MALT1^{-/+}$). $MALT1^{-/-}$ mice were completely protected from EAE, which was reflected in the absence of immune cell infiltration, demyelination and axonal damage in the spinal cord. Furthermore, splenocytes from $MALT1^{-/-}$ mice failed to produce an autoreactive T cell response and failed to induce autoimmune inflammation upon transfer in wild-type mice. Finally, cleavage of the MALT1 substrates A20 and CYLD was shown in wild-type T cells from EAE diseased mice. Collectively, these data demonstrate a crucial role for MALT1 in T cell activation and in the early priming phase of EAE, suggesting that targeting MALT1 might be an important therapeutic strategy to treat MS. Very recently, small compound MALT1 inhibitors that inhibit T cell activation and suppress the growth of the MALT1-dependent activated B cell subtype of diffuse large B cell lymphoma *in vitro* and *in vivo* have been described (Fontan *et al.,* 2012; Nagel *et al*., 2012). It will

therefore be of high interest to analyze the effect of such inhibitors on the development of EAE and other T cell mediated autoimmune pathologies (Mc Guire *et al*., in revision).

## Conclusion

With these studies, we hope to contribute to the better understanding of the pathways and molecular mechanisms that control autoimmune inflammation in the brain, and which are involved in the pathogenesis of MS and other neuroinflammatory conditions. This knowledge may have implications for the development of new therapeutics which may help in the treatment of patients suffering from these pathologies.

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Progress report of the research group of

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# From stem cells to cortical networks

## State of the Art / Objectives.

The cerebral cortex is one of the most complex and important structures in our brain. It contains dozens of different subtypes of neurons that are distributed into specific layers and areas, which allow efficient control of motor functions, sensory responses, as well as higher cognitive functions including language (Tiberi et al., 2012b).
The understanding of the mechanisms that generate this neuronal diversity and cortical cell-type specific properties could be instrumental to understand better human neurodevelopmental disorders such as mental retardation, autism and some forms of epilepsy. In addition, the ability to (re)specify cortical neurons in a controlled way could have a major impact for the rational design of brain repair strategies, to model its diseases, and for pharmaceutical screens.

We previously uncovered an intrinsic pathway of cortical neurogenesis, whereby mouse embryonic stem (mES) cells efficiently generate neurons that share most molecular, cellular and functional landmarks of pyramidal neurons of the cerebral cortex (Gaspard et al., 2009; Gaspard et al., 2008). This model opens new opportunities to study corticogenesis and its disorders.
Here we have followed a multidisciplinary research programme combining developmental neurobiology and pluripotent stem cell technology, centered on the mechanisms of cortical development in health and disease.

We focused on three main objectives:
***1. Understanding the mechanisms of specification of cortical neurons.***
***2. Linking development and evolution of the human cortex.***
***3. Exploring new ways to repair the diseased cortex.***

## Results

### 1 Understanding the mechanisms of specification of cortical neurons.

Using our model of corticogenesis from mouse ES cells, we have performed a gain of function screen aimed at identifying novel transcriptional programmes involved in cortical neurogenesis.

About 20 transcription factors known to be expressed in the developing cortex but without a well characterized function, were overexpressed transiently during in vitro cortical neurogenesis, using in lab-generated ESC lines allowing inducible gene expression upon doxycyclin (Dox) addition (Pietri et al., 2012; van den Ameele et al., 2012). Among the genes tested, the BCL6 B cell oncogene (Ye et al., 1997; Ye et al., 1993) stood up markedly as a potent proneurogenic factor, that triggers the differentiation of cortical progenitors into pyramidal neurons (Tiberi et al., 2012a).
To validate these findings physiologically, we tested for the requirement of BCL6 during normal in vivo cortical neurogenesis, using BCL6 knock-out mice. This revealed striking defects in cell cycle exit of neural progenitors and impaired production of pyramidal neurons following BCL6 gene disruption. These complementary approaches thus lead to the important conclusion that BCL6 is necessary and sufficient to promote the generation of cortical pyramidal neurons.
We then seeked for the molecular mechanism by which Bcl6 controls cortical neurogenesis, and found

that BCL6 acts by direct repression of the transcription of Hes5, an essential target of the Notch pathway that maintains cortical progenitors undifferentiated (Kageyama et al., 2009). In addition, we found that the repression of Hes5 by BCL6 involves the recruitment of Sirt1 NAD+-dependent deacetylase (Herranz and Serrano, 2010), which triggers Histone deacetylation at the level of the hes5 promoter, and thereby stable epigenetic silencing during neurogenesis.
These data identify BCL6 as a novel and key actor in cortical neurogenesis, and uncover for the first time Notch/BCL6/Sirt1 interactions that may impact many other aspects of physiology and disease (Tiberi et al., 2012a).

*This part of the project led to the following publications:*


- Tiberi L, van den Ameele J, Dimidschstein J, Piccirilli J, Gall D, Herpoel A, Bilheu A, Bonnefont J, Iacovino M, Kyba M, Bouschet T, and **Vanderhaeghen P**. 2012. BCL6 controls neurogenesis through Sirt1-dependent epigenetic repression of selective Notch targets. Nature Neuroscience 15:1627-1635.
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- van den Ameele J, Tiberi L, Bondue A, Paulissen C, Herpoel A, Iacovino M, Kyba M, Blanpain C, and **Vanderhaeghen P**. 2012. Eomesodermin induces Mesp1 expression and cardiac differentiation from embryonic stem cells in the absence of Activin. EMBO Reports 13:355-362.


## *2 Linking development and evolution of the human cortex.*

We have started to study the cellular and molecular properties of a novel model of corticogenesis from human ES cells recently developed in the lab, largely based on our previously described mouse ES model. Like in the mouse, human cortical-like progenitors are generated in a chemically defined medium, and cortical-like neurons (corresponding mostly to pyramidal neurons, the main subtype of cortical neurons) of diverse layers are generated in a time-dependent fashion. These pyramidal-like neurons can mature in vitro and make functional synapses with each other, as revealed by patch clamp experiments (Espuny-Camacho et al., 2013).
In addition, we performed xenografting of these human cortical cells into the neonatal cortex of NOD/SCID mice, to test for their capacity to integrate in cortical networks in vivo. This revealed that the transplanted neurons can integrate for up to 12 months in the mouse, and develop elaborate and specific patterns of axonal and dendritic projections. Furthermore electrophysiology experiments revealed that the transplanted neurons display functional synapses with each other, and with the host brain. These important data demonstrate for the first time in vivo the cortical identity of human ES/iPSC-derived neurons based on their morphology and pattern of connectivity with their subcortical targets.

Our results thus show that corticogenesis can be efficiently achieved from hES and iPS cells, following a pathway that is similar to its murine couterpart, but that also presents interesting differences, some of which may have direct relevance to brain evolution. Specifically, while mouse ES-corticogenesis takes about three weeks to be completed, it takes more than 10 weeks starting from human ES cells. In addition, the onset of neurogenesis appears much earlier in the mouse than in the human system, and is correlated with a different timing of appearance of neurogenic radial glia-like progenitors. Finally the timing of neuronal maturation is considerably slower with the human cells, as it takes up to six months for transplanted human neurons to develop mature patterns of axonal and dendritic growth, and synaptogenesis. Such differences are strikingly reminiscent of the properties of human corticogenesis (Fish et al., 2008; Kriegstein et al., 2006).

We have thus generated a unique experimental model to study cortical development with human cells (Espuny-Camacho et al., 2013), which can now be used to study developmental mechanisms related to human brain evolution (such as the srGAP2 gene uncovered in (Charrier et al., 2012), and to model human neurodevelopmental disorders.

*This part of the project led to the following publications:*

- Espuny-Camacho I, Michelsen K, Gall D, Linaro D, Hasche A, Bonnefont J, Bali C, Orduz D, Bilheu A, Herpoel A, Lambert N, Gaspard N, Péron S, Schiffmann SN, Giugliano M, Gaillard A, **Vanderhaeghen P**. 2013. Pyramidal neurons generated from human pluripotent stem cells integrate efficiently into mouse brain circuits in vivo. Neuron. 77:100-117.
- Charrier C, Joshi K, Coutinho-Budd J, Kim JE, Lambert N, de Marchena J, Jin WL, **Vanderhaeghen P**, Ghosh A, Sassa T, Polleux F. 2012. Inhibition of SRGAP2 Function by Its Human-Specific Paralogs Induces Neoteny during Spine Maturation. Cell. 149:923-935.

## 3 Exploring new ways to repair the diseased cortex.

We have started to explore the relevance of ESC-derived in vitro corticogenesis for brain repair, using intracerebral grafting in experimental models of cortical lesions in the mouse.
To achieve this, we first implemented a well established experimental setup (Gaillard et al., 2007): focal neuronal lesions of the cerebral cortex were generated following sterotactic injections of ibotenic acid neurotoxin, resulting in a focal loss of neurons in defined cortical domains, in frontal or occipital cortex. Three days after lesioning, Tau GFP ES-derived cortical progenitors and neurons (generated following (Gaspard et al., 2009) were grafted at the same site of the lesion.
Analysis of grafted animals 1-3 month after grafting indicated that most of them (80%) contained a graft, consisting mainly of differentiated pyramidal neurons. Most importantly, inspection of the rest of the brain revealed in 40% of the cases far-reaching graft-derived axonal growth, following specific paths and reaching specific targets of endogenous cortical neurons. Remarkably, we also found that the patterns of axonal growth were area-specific, i.e. ES-derived neurons with visual cortex identity and grafted in visual cortex send axons to visual and limbic targets, like in neonatal brain (Gaspard et al., 2008), but not following grafting in frontal cortex. These data indicate that ES-derived cortical neurons can display area-specific patterns of projections even in the adult brain, and that optimal restoration of cortical projections requires a precise match between the areal identity of the lesioned neurons and of the grafted neurons.

We next started to assess the functionality of the grafts using in vivo electrophysiology recordings. Specifically, in order to assess the potential of grafted ESC-derived cortical neurons for specific repair of the visual cortex, we tested whether they could be responsive to visual stimuli, using in vivo electrophysiology. These ongoing studies reveal that grafted ESC-derived cortical neurons display robust integration and functional properties similar to those of intact visual cortex, including responsiveness to physiological light stimulation. Collectively, these data constitute an important first step towards the rational study of pluripotent stem cell-derived neurons in brain repair strategies targeting the cortex.

*This part of the project led to the following publications:*

- Michelsen K, Acosta-Verdugo S, Benoit-Marand M, Espuny-Camacho I, Gaspard N, Saha B, Gaillard A, **Vanderhaeghen P**. 2012. Specific reestablishment of damaged circuits in the adult cerebral cortex by cortical neurons derived from mouse embryonic stem cells. Nature Neuroscience. in revision.

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## Publications resulting from work performed thanks to the FMRE/GSKE in 2012:


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