Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
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Queen Elisabeth Medical Foundation

Verslag – Rapport – Bericht - Report

2011

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

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Geneeskundige Stichting Koningin Elisabeth 2011
Inleiding verslag activiteiten van de GSKE – FMRE

Het jaar 2011 was de start van de nieuwe onderzoekteams geselecteerd door het wetenschappelijk comité en bevestigd door de Raad van Bestuur. Het wetenschappelijk comité had de moeilijke taak om de 65 ingediende projecten te klasseren. Niet alle goede projecten konden weerhouden worden omwille van de financiële moeilijkheden van de afgelopen jaren in ons land. De Raad van Bestuur heeft het aantal teams aanzienlijk moeten verminderen (van 17 naar 13) en heeft ook de jaarlijkse bedragen die toegekend worden aan elk onderzoeksteam moeten aanpassen. Dit alles is niet zo evident en daarom willen wij de leden van het wetenschappelijk comité en van de Raad van Bestuur danken voor het harde en ondankbare werk dat ze gerealiseerd hebben.

De driejaarlijkse cyclus blijft in principe gehandhaafd maar met een jaarlijkse evaluatie van de financiële mogelijkheden.

In de herfst, op 10 november, heeft de Raad van Bestuur bevestigd dat de onderzoekskredieten in 2012 behouden blijven op het niveau van 2011. Een nieuwe financiële evaluatie zal plaatsvinden eind 2012.

Op 16 juni vond de jaarlijkse prijsuitreiking plaats aan de geselecteerde onderzoeksteams voor hun werk van de afgelopen drie jaar: de prijs UCB aan professor Bart De Strooper van de KU Leuven, de Solvay Prize aan Professor Pierre Vanderhaeghen van de ULB, de ING prijs aan professor Serge Schiffmann van de ULB, de prijs Baron van Gysel de Meise aan professor Pierre Maquet van de ULg, de prijs Jacques en Janine Delruelle aan professor Wim Vanduffel van de KU Leuven, de prijs CBC Bank aan professor Christine Van Broeckhoven van de UA en de prijs Cercle Gaulois-Thierry Speeckaert aan professor Rufin Vogels van de KU Leuven. Na deze prijzen, was er ook de voorstelling van de onderzoeksteams die geselecteerd werden voor drie jaar. Het zijn de professoren Claudia Bagni en Danny Huylebroeck van de KU Leuven, de professoren Marc Cruts en Vincent Timmerman van de UA, de professor Fadel Tissir van de UCL, de professoren Christophe Ampe en Geert van Loo van de UGent, de professoren Eric Bellefroid, S.N. Schiffmann en Pierre Vanderhaeghen van de ULB, de professoren Laurent Nguyen en Pierre Maquet van de ULg en professor Ilse Smolders van de VUB.

Op 25 oktober bezocht Prinses Astrid samen met leden van de Stichting en Gravin de Laguiche, die de N.V Solvay vertegenwoordigde, het laboratorium van professor Pierre Vanderhaeghen, Solvay Prize 2011. De bezoeken van Prinses Astrid aan de laboratoria zijn zeer verrijkend zowel op wetenschappelijk als op menselijk vlak. Alle teamleden hebben de mogelijkheid om hun resultaten voor te stellen, van gedachten te wisselen en te dialogeren met de Prinses die altijd aandachtig luistert.

Het verslag van 2011 is ook een gelegenheid om de gulle schenkers 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 uit 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 bijdragen tot een goede werking de Geneeskundige Stichting Koningin Elisabeth.

Prof. em. dr. Baron de Barsy,
wetenschappelijk directeur
Brussel, december 2011

Fondation Médicale Reine Elisabeth
2011
Introduction rapport d'activités de la FMRE - GSKE

L'année 2011 a été l'année de mise en route des nouvelles équipes de recherche sélectionnées par le comité scientifique et désignées par le conseil d'administration. Le comité scientifique a eu la tâche difficile de classer les 65 projets qui lui avaient été soumis. Tous les bons projets n'ont pu être retenus en raison des difficultés financières connues dans notre pays depuis quelques années. Le conseil d'administration a été amené à réduire le nombre d'équipes de façon importante (de 17 à 13) et à procéder également à une réduction des montants accordés annuellement à chaque équipe. Tout ceci ne s'est pas fait sans peine et nous tenons à remercier très chaleureusement les membres du conseil scientifique et les membres du conseil d'administration pour le travail important et ingrat qu'ils ont dû réaliser.

Le cycle de trois ans est en principe maintenu, mais avec une réévaluation des possibilités financières chaque année.

En automne, le 10 novembre, le conseil d'administration a pu confirmer que les crédits 2012 seraient les mêmes que 2011. Une nouvelle évaluation des moyens financiers aura lieu fin 2012.

Le 16 juin a eu lieu la remise des prix aux équipes sélectionnées pour leur travail des trois années précédentes : il s'agit du prix UCB au Professeur Bart De Strooper de la KU Leuven, du prix Solvay au Professeur Pierre Vanderhaeghen de l'ULB, du prix ING au Professeur Serge Schiffmann de l'ULB, du prix Baron van Gysel de Meise au Professeur Pierre Maquet de l'ULg, du prix Janine et Jacques Delruelle au Professeur Wim Vanduffel de la KU Leuven, du prix CBC Banque au Professeur Christine Van Broeckhoven de l'UA et le prix Cercle Gaulois-Thierry Speeckaert au Professeur Rufin Vogels de la KU Leuven. Outre ces prix ,la séance prévoyait également la présentation des équipes sélectionnées pour 3 ans. Il s'agit des Prof. Claudia Bagni et Danny Huylebroeck de la KU Leuven, les Prof. Marc Cruts et Vincent Timmerman de l'UA, le Prof. Fadel Tissir de l'UCL, les Prof. Christophe Ampe et Geert van Loo de l'UGent, les Prof. Eric Bellefroid, S.N. Schiffmann et Pierre Vanderhaeghen de l'ULB, les Prof. Pierre Maquet et Laurent Nguyen de l'ULg et le Prof. Ilse Smolders du VUB.

Le 25 octobre la Princesse Astrid a rendu visite au laboratoire du Professeur Pierre Vanderhaeghen, lauréat du prix Solvay 2011, en compagnie de membres de la Fondation et de la comtesse de Laguiche, représentant la société Solvay. Ces visites de laboratoires menées par la Princesse Astrid sont particulièrement enrichissantes sur le plan scientifique, mais aussi sur le plan humain. Tous les membres de l'équipe ont l'occasion de présenter leurs résultats, d'échanger et de dialoguer avec la Princesse qui témoigne toujours d'une écoute et d'une attention soutenue.

Ce rapport de l'année 2011 est aussi l'occasion de remercier très chaleureusement les généreux mécènes qui ont choisis 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.

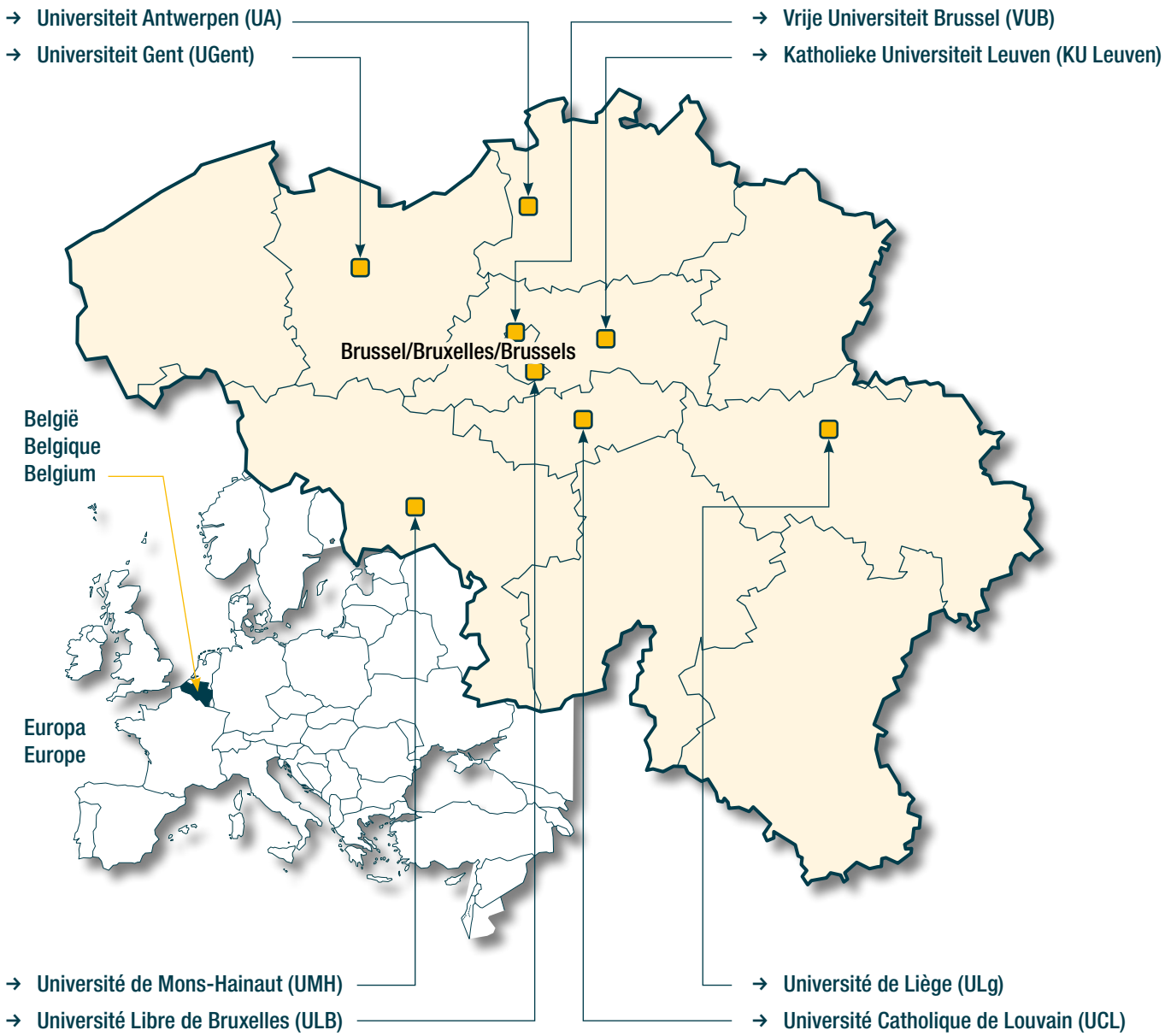
Nos remerciements vont également à la Princesse Astrid, notre présidente d'honneur, aux membres du conseil scientifique, aux membres du conseil d'administration et aux collaborateurs administratifs, qui acceptent de consacrer beaucoup de temps dans le cadre de leur compétence, au bon développement de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

Prof. em. dr. Baron de Barsy,
directeur scientifique
Bruxelles, décembre 2011

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. dr. Danny Huylebroeck**
  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**
  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

- **Prof. 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**
  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.

- **Prof. 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 (2011)

Progress report of the research group of

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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: ß- and γ- cytoplasmic actin (Tondeleir *et al.*, 2009). Their differential roles are unknown and it was long time accepted these isoforms were redundant because they only differ in four amino acids. Recent studies employing mice show, however, these isoforms are not redundant. γ-actin knock-out (KO) mice are viable but show progressive hearing loss during adulthood, despite compensatory up-regulation of ß-actin (Belyantseva *et al*., 2009) whereas ß-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.*, in press). Clearly the increased expression of other actin isoforms fails to rescue the lethal phenotype and does not compensate for lack of ß-actin. Additionally expressing γ-actin from the ß-actin locus does not rescue the lethal phenotype although occasionally embryos survive until E13.5-14.5 suggesting partial rescue (Lambrechts *et al.*, unpublished).

ß-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. 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 ß (TGF-ß) 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.* in press).

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

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

We exploit the ß-actin knock-out mouse model. Consistent with defective MEF migration, we observed in ß-actin KO embryos phenotypes that can be attributed to impaired cell migration: e.g. aberrant vascularization (requiring endothelial cell migration) and, relevant for this project, an amazing lack of neurons (requiring neural crest cell migration, NCC) (**Figure 1**).

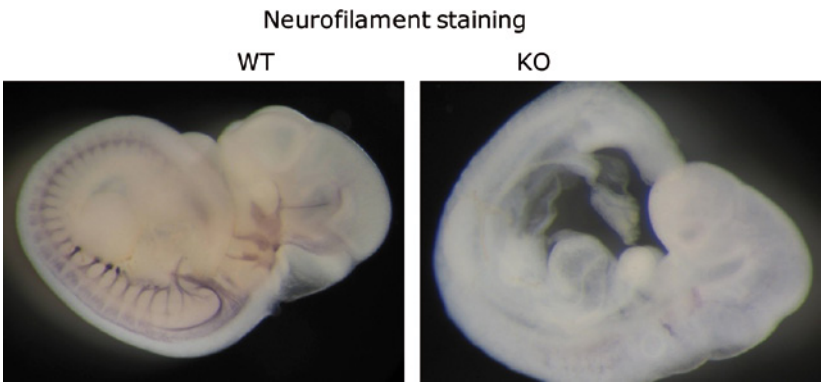
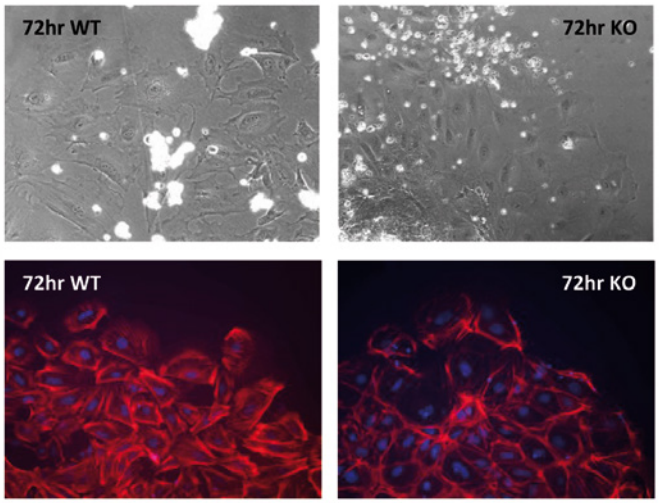


Figure 1

Using sox10, a marker for migratory NCCs we showed that the neural crest population is specified, but subsequent delamination/migration steps are affected in the ß-actin knock-out embryos. Explanting the neural tubes onto fibronectin *in vitro* confirms the observed phenotype *in vivo*: ß–actin KO NCCs leave the neural tube in less abundant numbers compared to WT NCCs. In addition, after 72 hours, the emigrated NCCs leave as an epithelial sheet, in close contact with their neighbours. Unlike WT cells they almost never become single cells that migrate far away from the explant (**Figure 2**).

Figure2



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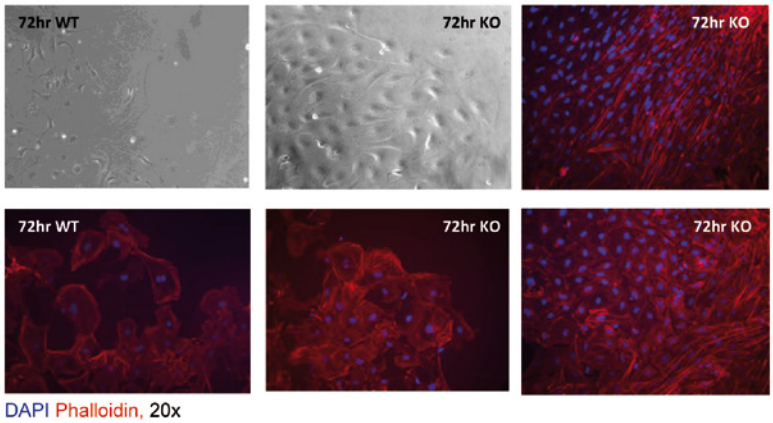
Filamentous (F)-actin distribution, monitored by phalloidin staining, is very different in WT versus KO explants. WT outgrowths display leading edge cells with large lamellipodia and high stress fiber content, mostly positioned in parallel with the direction of movement. KO outgrowths do not display cells with such large lamellipodia, nor do they have this high amount of central stress fibers. F-actin is mostly positioned as cortical actin, forming a ring around the cell periphery. Based on our work with MEFs, where we could restore cell migration with ROCK inhibitor (Tondeleir *et al.*, in press), we applied this chemical drug after explanting the neural tubes. In this experiment, adding ROCK inhibitor mainly affected the epithelial sheet around the KO tube, becoming very broad. Only a limited amount of cells adopted spindle shaped morphology as single cells. This was much more the case for WT explants, where the emigration surface became much wider. F-actin distribution also changed dramatically (**Figure 3**). The WT leading cells lose their prominent stress fibers (as expected)

Figure3



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and lamellipodia, and actin becomes more diffusely organised. The leading edge cells of the KO explant starts to resemble the leading edge cells of the WT explants treated with ROCK inhibitor, they switch their prominent cortical actin for more diffusely organised actin. The broad epithelial sheet in the KO explant treated with ROCK inhibitor is characterised by very compact cells (**Figure 3**). These results indicated that, in contrast to ß-actin KO MEFs, inhibiting ROCK affects NCC migration behaviour only to a limited extent and this was insufficient to achieve normal emigration of ß-actin KO NCCs *ex vivo*. Therefore other signalling pathways are likely involved. An obvious candidate is the TGFß pathway that cross-talks with Rho (this will be investigated in 2012) and, for reasons outlined immediately below, the cadherin signalling pathway.

Given the observed phenotype (lack of single cell migration and behaviour as a sheet) and given the importance of cadherins in cell-cell interactions and the connection with actin cytoskeleton it is worth paying attention to cadherin switching which occurs during NCC EMT prior to delamination (Acloque *et al.*, 2009). We started to examine cadherin expression during neural crest ontogony (poorly documented for mouse in literature) and are mapping cadherin expression at stage E10,5 in WT mice using immune-histochemistry. Once protocols and probes for the various investigated cadherins (E-, N-cadherin, cadherins 6 and 7) have been optimized we will expand this to cadherin expression at multiple stages during neural crest ontogeny (E8,5-10,5). Thus we will be able to identify possible cadherin misregulations in the ß-actin knockout embryos and this will enable us further unravelling molecular signaling involved in the impaired neural crest delamination/migration.

B The role of the 3'UTR of ß-actin transcript in neurogenesis.

The 3'UTR of ß-actin mRNA contains the so-called zip-code: a 54 nucleotide sequence immediately 3' of the ß-actin stop codon. This region interacts with zipcode binding protein 1 (Zbp1) which controls both transport and translation of the ß-actin mRNA (Condeelis and Singer, 2005; Huttelmayer *et al.*, 2005). Down-stream of the zipcode is a region required for stability of the mRNA and this region interacts with the RNA binding protein Hur (Dormoy-Raclet *et al.*, 2007). The 3'UTR of ß-actin mRNA is known to be important for neuritogenesis and growth cone steering down-stream of neurotrophic or chemotropic factors (Leung *et al.*, 2006, Yao *et al.*, 2006). Mice expressing human (h) ß-actin cDNA without the entire 3' UTR (*Actb*$^{hACTB/hACTB}$) are more agitated, suggesting a behavioral problem, and develop enlarged spleens (Tondeleir *et al.*, unpublished result).

In order to characterize defects in brain of this mouse, as originally proposed in the application, we set up a breeding programme to compare brains of *Actb*$^{+/+}$, *Actb*$^{+/hACTB}$, *Actb*$^{hACTB/hACTB}$, *Actb*$^{-/hACTB}$ and *Actb*$^{+/GFP}$ mice. Since the murine and human ß-*actin* gene sequences are very similar time was devoted to develop a highly specific PCR screen distinguishing these two DNAs. Employing this screen in the breeding program we observed very few homozygous *hACTB* knock in offsprings and viable heterozygous (*Actb*$^{GFP/hACTB}$) newborns were never obtained, whereas at stage E10,5 such embryos are present. This could suggest that haploinsufficiency of (human) ß-actin leads to embryonic lethality, however this results is peculiar since heterozygous mice (*Actb*$^{+/GFP}$) are viable and have only slightly reduced ß-actin protein levels (Tondeleir *et al.*, in press). The difference in phenotype may thus rather be due to the absence of regulatory elements in the 3'UTR in the transgene expressing hß-actin (human and mouse ß-actin are identical in protein sequence). Given that the ß-actin mRNA is an abundant one, this may result in a disturbance of the balanced regulation of other genes involved in neurogenesis. Indeed a genome wide identification of targets of Zbp family members was published (Hafner *et al.*, 2010). An *in silico* analysis emphasizes the importance of Zbp1 in nervous system development and function (43 mRNAs, appr. 10% of identified specific targets) and in particular in axonal guidance signaling (16 known specific targets) whereas specific targets for the other two members of the Zbp family are not significantly enriched in these processes (Ampe *et al.*, unpublished).

The unforeseen difficulty in breeding hampers the planned behavioral studies at the German mouse clinic (www.mouseclinic.de) because a sufficient number of littermates of the various phenotypes (see above) need to be compared in parallel. In addition, just after the start of the funding a paper from a competing group appeared showing that ß-actin does not play a role in motor neuron viability and maintenance (the Cre system used precluded observing early defects during neuronal growth, Cheever *et al.*, 2011). Our observation that ß-actin knock out embryos have no neurons (**Figure 1**) suggest however that early phases of neurogenesis are dramatically affected. We therefore will refocus the

research entirely on early developmental defects (including NCC migration, see above) also using the *Actb*$^{hACTB/hACTB}$ mouse model as we expect it will allow to investigate the importance of the 3'UTR of ß-actin in crosstalk to other molecules with a neuronal function (such as fragile X mental retardation protein 1 that also interacts with Zbp1, Rackham and Brown, 2004).

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Progress report of the research group of

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

1. Research program summary

Memory formation and cognitive processes that rely on activity-dependent synaptic plasticity are affected by local protein synthesis. Synaptic inputs dictate the time, place and amount of protein synthesis necessary for the single synapses. Dysregulation of these mechanisms leads to spine dysmorphogenesis 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. 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. In human, CYFIP1 gene is also associated to Autism and Schizophrenia. 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 stimulation. This project will shed light into mental retardation, Autism and possibly schizophrenia.

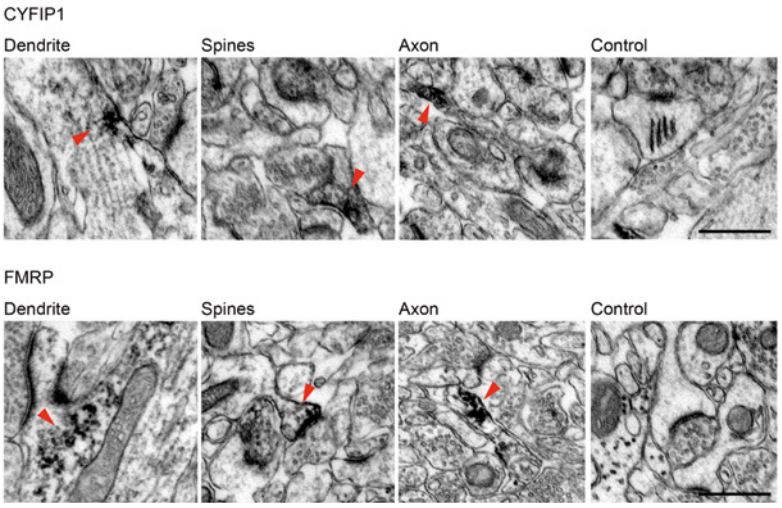
2. Outcome

2.1 Achievements

We believe that CYFIP1 has a critical role in important synaptic processes, and therefore unbalances in CYFIP1 interactive network might result in neuronal dysfunctions as the dendritic spines dysgenesis observed in patients with Fragile X Syndrome and possibly other mental disorders.
For these reasons, we have deepened the current knowledge on CYFIP1 synaptic complex/es formation and 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) (see Figure on the left, red arrows), and immunohistochemistry (IHC) studies that indicate that CYFIP1 and FMRP are enriched at synapses. We next focused on this



highly specialized subcellular compartment which is largely affected in several neurological syndromes and in mental retardation.

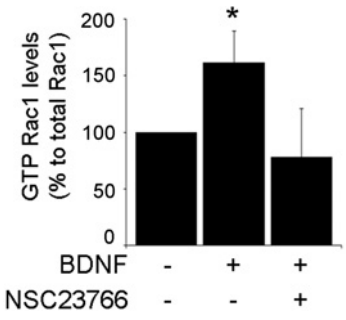
Therefore, the CYFIP1 complexes were isolated by IP/MS on cortical lysates and purified synapses (synaptoneurosomes). We identified 35 co-interacting proteins, including RNA-binding proteins implicated in mRNA metabolism and actin cytoskeleton regulators. Intriguingly, the composition of the complexes changes according to the subcellular localization. In particular, the synaptic CYFIP1 interactome is distributed in two main complexes: 1) CYFIP1-FMRP-eIF4E, implicated in translational control and 2) the WAVE Regulatory Complex (WRC), involved in remodeling of the actin cytoskeleton.

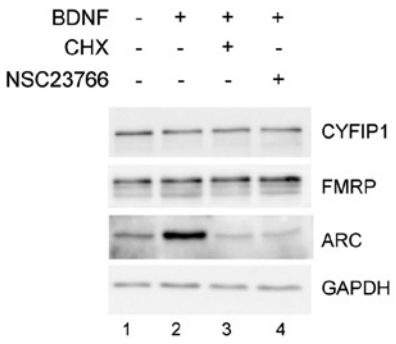
Since CYFIP1 has been related to intellectual disability, autism and schizophrenia, we investigated whether the genes encoding the CYFIP1 partners are involved in neuropsychiatric disorder by disease annotation based on published literature. Importantly, we have found that 2/3 of the novel CYFIP1 interactors show a significant association with intellectual disability, autism and schizophrenia.

Future plans 1: We plan to further deepen the disease involvement of CYFIP1 partners by candidate gene-based analysis for association with autism and schizophrenia; for this purpose, we will interrogate meta-analysis data performed by Genome-Wide Association Study (GWAS) Consortia, in collaboration with Dr. Danielle Posthuma, Department of Functional Genomics and Department of Medical Genomics, VU Amsterdam, The Netherlands. Additionally, we plan in the future to study the expression profile of several CYFIP1 interactors in post mortem brain tissues from patients with autism and schizophrenia. We are currently gathering brain specimens from publicly available databanks, such as the Stanley Medical Research Institute, which will provide purified proteins from cingulate, frontal cortex and cerebellum from schizophrenia patients and unaffected controls.

Aim 2: *Investigate whether actin cytoskeleton regulators (i.e. the Rho GTPases) affect local protein synthesis mediated by CYFIP1.*

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 Rac1, a small GTPase upstream of the WRC, might release CYFIP1 from eIF4E and relocating it on the WRC. We have so far direct *in vitro* evidence that active, GTP-bound Rac1 competes with eIF4E for the interaction with CYFIP1. Furthermore, we are testing the ability of a lipophilic Rac1 inhibitor (NSC23766) of modulating CYFIP1-eIF4E interaction and the translation dependent upon this complex. For this purpose, we have first verified that NSC23766 was able to prevent the activation of Rac1 in response to translational-inducing stimuli, i.e. the neurotrophin BDNF, in primary cortical neurons (see Figure on the left).

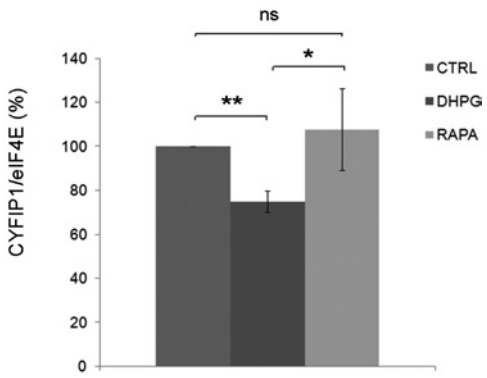




Notably, we found that inhibiting Rac1 activation by NSC23766 administration prevents the BDNF-induced translation of CYFIP1-FMRP targets mRNAs, i.e. *Arc* mRNA (see Figure on the right).

Future plans 2: To further corroborate the hypothesis that the activation of Rac1 induces the dissociation of CYFIP1-eIF4E, we will directly study the dynamics of CYFIP1-eIF4E interaction on synaptoneurosomes stimulated with BDNF with or without NSC23766 pre-treatment. Furthermore, we will study whether the effects of NSC23766 on BDNF-induced ARC synthesis are dependent on CYFIP1 and/or FMRP by performing the assay in primary cortical neurons silenced for Cyfip1 or lacking FMRP (*Fmr1* KO).

Aim 3: *Study the physiological regulation of FMRP and CYFIP1 upon synaptic stimulation at synapses.*
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 signalling cascade affects not only general 4E-BPs, but also specific one as CYFIP1, we stimulated acute hippocampal slices with a group I mGluRs DHPG and we simultaneously block mTORC1 with rapamycin. CYFIP1-eIF4E association was then investigated by m7GTP chromatography and 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 (see Figure on the right). To further support these data, we have collected mouse brain tissues from a transgenic mouse line with enhanced activation of mTORC1 (FKBP12 KO), provided by Prof. Eric Klann, NYC, USA.



Future plans 3: We plan to study the phosphorylation state of CYFIP1 by phosphoproteomics, in collaboration with Prof. August B. Smit, Dept. of Molecular and Cellular Neurobiology, Center for Neurogenomics and Cognitive Research, VU University Amsterdam, The Netherlands. For this purpose, we will immunoprecipitating CYFIP1 from unstimulated and DHPG-treated synaptoneurosomes and analyzing the presence of phosphopeptides by MS.In addition to gath conclude the phosphoproteomics. Furthermore, since translational control and actin cytoskeleton rearrengements are very important for dendritic spine morphology, we plan to investigate 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.

2.2 Networking and collaborations

The project results from the integration of complementary expertise, giving us the opportunity to create productive scientific collaborations in our Institute and with 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, we will collaborate 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 of the Center of Human Genetics, KU Leuven (Light Microscopy & Imaging Network, LiMoNe) for spine visualization and analysis.

3. Relevance

The work performed so far greatly contributed to the current knowledge of CYFIP1 distribution and function/s in brain. The composition of the molecular complexes we have identified in this study is completely novel and foundamental to start unraveling the different, interconnected CYFIP1 cellular functions. Furthermore, our data shed light on the regulatory mechanisms tuning CYFIP1 complexes with neuronal stimulation. This integrated approach is of utmost importance not only in the context of Fragile X Syndrome and other intellectual disabilities form, but also in the fields of autism and schizophrenia. Furthermore, the isolation of protein interactomes with a significant pathological involvement helps

understanding the interconnection and co-morbidity between different neuropsychiatric disorders. Considering the novel and intriguing results obtained so far, we believe that a further support of a year from *Fondation Médical Reine Elisabeth* will strongly help us in completing this research and gave further insights into Fragile X Syndrome, autism and schizophrenia.

4. Publications in preparation/submitted

- De Rubeis S, Li KW, Buzzi A, Pasciuto E, Yang B, Di Marino D, Fernandez E, Ostroff LE, Zwartkruis F, Eric Klann[4], Komiyama NH, Grant S, Posthuma D, Smit AB and Bagni C. The Cytoplasmic FMRP Interacting Protein 1 links mRNA translation to spine remodeling: insights into Autism and Schizophrenia. *Will be submitted to Cell next month.*
- De Rubeis S, Férnandez E, Li KW, Smit AB, Bagni C. New insights in the FMRP role in the regulation of PSD-95 mRNA stability. *In preparation.*

5. Team key publications (last five years).

- Lacoux C, Di Marino D, Pilo Boyl P, Zalfa F, Yan B, Ciotti MT, Falconi M, Urlaub H, Achsel T, Mougin A, Caizergues-Ferrer M, **Bagni C.** (2012) BC1-FMRP interaction is modulated by 2'-Omethylation: RNA-binding activity of the tudor domain and translational regulation at synapses. *Nucleic Acids Res.* [Epub ahead of print]
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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 Dmrt5 gene encodes a doublesex homolog zinc finger transcription factor we found to be expressed in a graded manner within the cortical neuroepithelium. During this year, we pursued the analysis of the phenotype of the constitutitve knockout mouse we generated in the laboratory. Ou results show 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 is currently under revision for the revue *Cerebral cortex* (see **annexe 1**).

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 show 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 is currently submitted for publication to the journal *Mol. Cell Biol* (see **annexe 2**).

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- Parlier D., Moers V., Preillon J., Van Campenhout C., Marine J-C., Bellefroid E.J. The *Xenopus* doublesex-related gene *xDmrt5* is required for olfactory placode neurogenesis. Submitted to ***Mol. Cell Biol.***
- Mazurier N., Parlier D., Parain K., Pretto S., Locker M., Bellefroid E.J., Perron, M. Ascl1 as a novel player in the Ptf1a transcriptional network for GABAergic cell specification in the retina. Submitted to ***Development***
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Progress report of the research group of

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Molecular Genetics and Functional Genomics of Frontotemporal Lobar Degeneration

Specific Aims

In this research project we aimed to further 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
1. extend our FTLD patient and matched control samples
2. identify genes modifying onset age in FTLD
3. identify novel causal genes for FTLD
4. identify novel susceptibility genes for FTLD

In 2011, the main focus was on objectives 1, 3 and 4. The major achievement of 2011 was the identification of a hexanucleotide repeat expansion in the C9orf72 gene in the ALSFTD2 locus of chromosome 9p (Gijselinck et al., 2012b) as a major cause of inherited FTLD with or without symptoms of motor neuron disease, of which up to 30% is explained by this mutation. Moreover, the repeat expansion turns out to be an equally important cause of amyotrophic lateral sclerosis. The progress we made towards the research objectives are reported.

Novel causal genes for FTLD

In 2006, we identified *GRN* null mutations as a major gene for FTLD explaining 26 % of familial FTLD in Belgium (Cruts et al., 2006). Following up on this, we studied in detail the clinical and genetic characteristics of patients with *GRN*-associated FTLD. As a first step towards therapeutics, we identified agents that have the potential to increase *GRN* expression back to normal levels in these patients.

In 2010, we reported linkage data in an extended FTLD-ALS family to chromosome 9p21 (Gijselinck et al., 2010). Now, we identified a hexanucleotide repeat expansion in the promoter of the *C9orf72* gene as the cause of disease in this family. It is the most commonly mutated gene in FTLD and also in ALS.

Granulin (*GRN*)

A 46-site International FTLD Collaboration was formed to collect cases of FTLD with TAR DNA-binding protein of 43-kDa (TDP-43)-positive inclusions (FTLD-TDP). We identified 97 individuals with FTLD-TDP with pathogenic *GRN* mutations (GRN+ FTLD-TDP), assessed their genetic and clinical characteristics, and compared them with 453 patients with FTLD-TDP in which *GRN* mutations were excluded (GRN- FTLD-TDP). No patients were known to be related. Neuropathologic characteristics were confirmed as FTLD-TDP in 79 of the 97 GRN+ FTLD-TDP cases and all of the GRN- FTLD-TDP cases. Age at onset of FTLD was younger in patients with GRN+ FTLD-TDP vs GRN- FTLD-TDP (median, 58.0 vs 61.0 years; $P < .001$), as was age at death (median, 65.5 vs 69.0 years; $P < .001$). Concomitant motor neuron disease was much less common in GRN+ FTLD-TDP vs. GRN- FTLD-TDP (5.4% vs 26.3%; $P < .001$). Fifty different *GRN* mutations were observed, including 2 novel mutations: c.139delG (p.D47TfsX7) and c.378C>A (p.C126X). The 2 most common *GRN* mutations were c.1477C>T (p.R493X, found in 18

patients, representing 18.6% of *GRN* cases) and c.26C>A (p.A9D, found in 6 patients, representing 6.2% of cases). Patients with the c.1477C>T mutation shared a haplotype on chromosome 17; clinically, they resembled patients with other *GRN* mutations. Patients with the c.26C>A mutation appeared to have a younger age at onset of FTLD and at death and more parkinsonian features than those with other *GRN* mutations (Chen-Plotkin et al., 2011).

Consistent with the observation that GRN has neurotrophic properties, pharmacological stimulation of GRN production is a promising approach to rescue *GRN* haploinsufficiency and prevent disease progression. We therefore searched for compounds capable of selectively increasing GRN levels. Here, we demonstrate that four independent and highly selective inhibitors of vacuolar ATPase (bafilomycin A1, concanamycin A, archazolid B, and apicularen A) significantly elevate intracellular and secreted GRN. Furthermore, clinically used alkalizing drugs, including chloroquine, bepridil, and amiodarone, similarly stimulate GRN production. Elevation of GRN levels occurs via a translational mechanism independent of lysosomal degradation, autophagy, or endocytosis. Importantly, alkalizing reagents rescue GRN deficiency in organotypic cortical slice cultures from a mouse model for GRN deficiency and in primary cells derived from human patients with *GRN* loss-of-function mutations. Thus, alkalizing reagents, specifically those already used in humans for other applications, and vacuolar ATPase inhibitors may be therapeutically used to prevent *GRN*-dependent neurodegeneration (Capell et al., 2011).

C9orf72

We had identified conclusive genetic linkage to a locus on chromosome 9p13 21 in one FTD-MND family, family DR14 (Gijselinck et al., 2010). Worldwide, 13 families segregating both FTLD and MND were linked to this chromosomal region (Gijselinck et al., 2012a), suggesting that it is the major locus for ALS-FTD (**Figure 1)**.

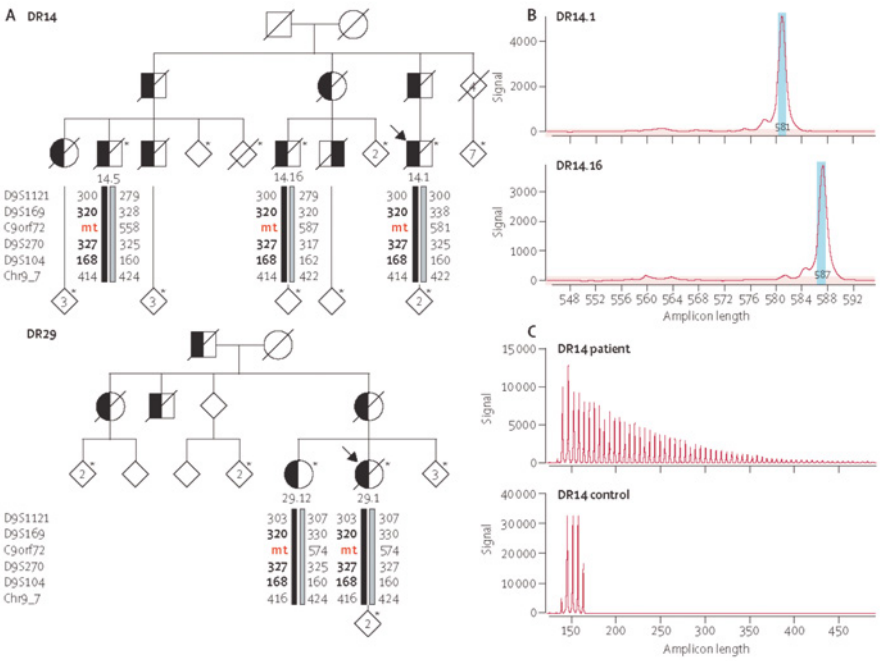

Figure 1 *C9orf72* repeat expansion in FTLD-ALS (**A**) Abbreviated pedigrees of genetically related families DR14 and DR29. Disease haplotypes segregating in the families are shown as black bars and the haplotype shared between families DR14 and DR29 are shown in bold. The *C9orf72* repeat is included in the haplotype with mt representing the repeat expansion on the mutant allele. Sex, birth order, and mutation status of unaffected individuals have been omitted for reasons of confidentiality. (**B**) Chromatograms of the *C9orf72* repeat in two patients of family DR14 showing absence of segregation resulting from hemizygosity of the normal allele owing to loss of the mutant allele with the *C9orf72* repeat expansion. (**C**) Results from one patient and one control in family DR14 with repeat-primed PCR amplification assay showing the GGGGCC repeat expansion as multiple peaks (Gijselinck et al., 2012a).

We studied 305 patients with FTLD, 137 with amyotrophic lateral sclerosis (ALS), and 23 with concomitant FTLD and ALS (FTLD-ALS) and 856 control individuals from Flanders (Belgium). Patients were identified from a hospital-based cohort and were negative for mutations in known FTLD and ALS genes. We also examined family DR14 of one patient with FTLD-ALS previously linked to 9p21. We analysed 130 kbp at 9p21 in association and segregation studies, genomic sequencing, repeat genotyping, and expression studies to identify the causal mutation. We compared genotype-phenotype correlations between mutation carriers and non-carriers. In the patient-control cohort, single-nucleotide polymorphism rs28140707 within the 130 kbp region of 9p21 was associated with disease (odds ratio [OR] 2.6, 95% CI 1.5-4.7; p=0.001). A GGGGCC repeat expansion in *C9orf72* completely co-segregated with disease in family DR14. The association of rs28140707 with disease in the patient-control cohort was abolished when we excluded GGGGCC repeat expansion carriers. In patients with familial disease, six (86%) of seven with FTLD-ALS, seven (47%) of 15 with ALS, and 12 (16%) of 75 with FTLD had the repeat expansion. In patients without known familial disease, one (6%) of 16 with FTLD-ALS, six (5%) of 122 with ALS, and nine (4%) of 230 with FTLD had the repeat expansion. Mutation carriers primarily presented with classic ALS (10 of 11 individuals) or behavioural variant FTLD (14 of 15 individuals). Mean age at onset of FTLD was 55.3 years (SD 8.4) in 21 mutation carriers and 63.2 years (9.6) in 284 non-carriers (p=0.001); mean age at onset of ALS was 54.5 years (9.9) in 13 carriers and 60.4 years (11.4) in 124 non-carriers. Postmortem neuropathological analysis of the brains of three mutation carriers with FTLD showed a notably low TDP-43 load. In brain at postmortem, C9orf72 expression was reduced by nearly 50% in two carriers compared with nine controls (p=0.034). In familial patients, 14% of FTLD-ALS, 50% of ALS, and 62% of FTLD was not accounted for by known disease genes. The GGGGCC repeat expansion is highly penetrant, explaining all of the contribution of chromosome 9p21 to FTLD and ALS in the Flanders-Belgian cohort. Decreased expression of *C9orf72* in brain suggests haploinsufficiency as an underlying disease mechanism (Gijselinck et al., 2012b).

Novel susceptibility genes for FTLD

in 2010, the first genome-wide association (GWA) study in frontotemporal lobar degeneration (FTLD) identified common genetic variability at the *TMEM106B* gene on chromosome 7p21.3 as a potential important risk-modifying factor for FTLD with pathologic inclusions of TAR DNA-binding protein (FTLD-TDP), the most common pathological subtype in FTLD (Van Deerlin et al., 2010). To gather additional evidence for the implication of *TMEM106B* in FTLD risk, multiple replication studies in geographically distinct populations were set up. Our and other recent replication and follow-up studies of the FTLD-TDP GWA study and provided a growing body of evidence that establish *TMEM106B* as a bona fide risk factor for FTLD. With the *TMEM106B* gene, a new player has been identified in the pathogenic cascade of FTLD which could hold important implications for the future development of disease-modifying therapies (van der Zee and Van Broeckhoven, 2011). Further, we studied the spinocerebellar ataxia gene ataxin 2 (*ATXN2*) that was associated with ALS (Elden et al., 2010) in ALS patients and, for the first time, evaluated its contribution to FTLD.

TMEM106B

In a genome-wide association study of frontotemporal lobar degeneration with pathological inclusions of TAR DNA-binding protein, significant association was obtained with three single nucleotide polymorphisms at 7p21.3, in a region encompassing the gene *TMEM106B*. This study also suggested a potential modifying effect of *TMEM106B* on disease since the association was strongest in *GRN* mutation carriers. Further, the risk effect seemed to correlate with increased *TMEM106B* expression

in patients. In the present study, we sought to replicate these three findings using an independent Flanders-Belgian cohort of primarily clinically diagnosed patients with FTLD (n = 288). We were able to confirm the association with *TMEM106B* with a P-value of 0.008 for rs1990622, the top marker from the genome-wide association study [odds ratio 0.75 (95% confidence interval 0.61-0.93)]. Further, high-density SNP mapping suggested that the association was solely driven by the gene *TMEM106B*. Homozygous carriers of the *TMEM106B* protective alleles had a 50% reduced risk of developing FTLD. However, we were unable to detect a modifying effect of the *TMEM106B* SNP on onset age in *GRN* mutation carriers belonging to an extended, clinical and pathological well-documented founder family segregating a *GRN* null mutation. Also, we could not observe significant differences in mRNA expression between patients and control individuals in lymphoblast cell lines and in brain frontal cortex (van der Zee et al., 2011).

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., 2011).

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Administrative Report

Honors, Prizes & Awards

Fellowships

International

– **G. Kleinberger:** European Molecular Biology Organization, EMBO Short-Term fellowship, Research Visit, Munich, Germany, February 21 – May 21, 2011

Travel Awards

International

– **T. Van Langenhove:** Alzheimer's Association, AA Travel Award, Alzheimer's Association International Conference 2011, Paris, France, June 17-21, 2011

National

– **I. Gijselinck:** Research Foundation – Flanders (FWO), FWO Travel Award for participation to an international meeting: "International congress of human genetics", Montreal, Canada, October 11-15, 2011

Organisations & Presentations

Chair and Organizational Activities

– **van der Zee J.:** 15th congress of the European Federation of Neurological Societies (EFNS2011), poster session Ageing and dementia 2, Budapest, Hungary, September 10-13, 2011, Chair

Invited Lectures

International

– **Cruts M.**: 'Molecular genetics of frontotemporal lobar degeneration ', Annual Congress of the Deutschen Geselschaft für Neurologie 2011, Wiesbaden, Germany, September 28 – October 1, 2011
– **van der Zee J.:** 'Recent advances in genetics of early onset dementia', Luigi Amaducci teaching course on dementia – advanced, 15th congress of the European Federation of Neurological Societies, Budapest, Hungary, September 10-13, 2011

National

– **van der Zee J.**: 'Follow-up of genome-wide association studies in a powerful study population of Belgian FTLD patients' International Alzheimer Research Foundation (SAO/FRMA) – Alzheimer Day, Kasteel van Groot Bijgaarden, Brussels, Belgium, September 18, 2011

Oral Presentations – Slide Sessions

International

– **Philtjens S.:** 'Mutation detection using whole genome sequencing in frontotemporal lobar degeneration', Mutation Detection 2011, Santorini, Greece, June 6-10, 2011
– **Van Langenhove T.:** 'Association of intermediate-length poly-Q expansions in ATXN2 with ALS but not FTLD in a Flanders-Belgian cohort', Alzheimer's Association, Paris, France, July 16-21, 2011

National

– **Gijselinck I.:** 'Expansion of a G4C2 repeat sequence in the C9orf72 promoter is a common cause of both familial and sporadic FTLD and ALS disorders in Flanders-Belgium', IAP P6/43 Meeting, Sint-Lambrechts-Woluwe, Belgium, October 24, 2011
– **Pereson S.:** 'Progranulin beneficially modifies Alzheimer disease-related pathology in APPPS1 mice', IAP P6/43 Meeting, Sint-Lambrechts-Woluwe, Belgium, October 24, 2011

Poster Presentations

International

– **Gijselinck I.:** 'Identification of novel genes for frontotemporal lobar degeneration using whole genome sequencing', 12th International Congress of Human Genetics (ICHG2011), Montréal, Canada, October 11-15th, 2011
– **Janssens J.:** 'Overexpressing mutant M337V human TDP-43 causes a dose-dependent ALS motor neuron phenotype in transgenic mice', Fondation IPSEN, Paris, France, May 9, 2011
– **Janssens J.:** 'Mutant human TDP-43 overexpression in mice leads to a dose-dependent ALS motor neuron phenotype', 8th IBRO World Congress of Neuroscience, Florence, Italy, July 14-18, 2011
– **Kleinberger G.:** 'In vitro studies of the pathogenic effect of missense mutations on key biological properties of progranulin associated with CNS neurodegeneration', 8th IBRO World Congress of Neuroscience, Florence, Italy, July 14-18, 2011
– **van der Zee J.:** 'Pursuit of the biological variant underlying the association of TMEM106B with frontotemporal lobar degeneration', 12th International Congress of Human Genetics (ICHG2011), Montréal, Canada, October 11-15th, 2011

National

– **Janssens J.:** 'Mutant human TDP-43 overexpression accelerates core disease features of FTLD-ALS in a novel mouse model compared to wild-type human TDP-43 overexpression mice', IAP P6/43 Meeting, Sint-Lambrechts-Woluwe, Belgium, October 24, 2011
– **Philtjens S.:** 'Unraveling the genetic etiology of frontotemporal lobar degeneration using whole genome sequencing', IAP P6/43 Meeting, Sint-Lambrechts-Woluwe, Belgium, October 24, 2011

Societal activities

– **van der Zee J.:** 'De Ziekte van Alzheimer, nieuwe wetenschappelijke inzichten rond dementie', Vlaamse Alzheimer Liga en Vormingplus Gent-Eeklo, Knesselare, Belgium, March 21, 2011
– **van der Zee J.:** 'Dementie en het genetisch onderzoek', KVLV, Deinze, Belgium, November 9, 2011
– **Van Langenhove T.:** 'Casus patient with GRN mutation and genetics of FTLD in Flanders-Belgium', Dementieclub geriatrie Oost en West-Vlaanderen, Zwijnaarde, Belgium, October 19, 2011

Publications acknowledging G.S.K.E. funding

Articles in International Journals

- **van der Zee,J.**, Van Langenhove,T., Kleinberger,G., Sleegers,K., Engelborghs,S., Vandenberghe,R., Santens,P., Van den Broeck,M., Joris,G., Brys,J., Mattheijssens,M., Peeters,K., Cras,P., De Deyn,P.P., **Cruts,M.**, Van Broeckhoven,C.: TMEM106B is associated with frontotemporal lobar degeneration in a clinically diagnosed patient cohort. Brain 134: 808-815 (2011) (PMID: 21354975) (I.F.: 9.23)
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- **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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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

- Van Langenhove,T., Engelborghs,S., Vandenberghe,R., Santens,P., Cras,P., Nuytten,D., De Jonghe,P., De Deyn,P., **Cruts,M.**, Van Broeckhoven,C., **van der Zee,J.**: Association of intermediate-length polyQ expansions in ATXN2 with ALS but not FTLD in a Flanders-Belgian cohort Alzheimer's Association International Conference on Alzheimer's Disease 2011 (ICAD 2010), Porte de Versailles, Paris, France, July 16-21 7(4 Supp1): S685 (2011)
- Janssens,J., Wils,H., Kleinberger,G., Joris,G., Cuijt,I., Kumar-Singh,S., Van Broeckhoven,C.: D421 mutant human TDP-43 overexpression in mice leads to a dose-dependent ALS motor neuron phenotype. 8th IBRO World Congress of Neuroscience, Florence, Italy, July 14-18 : D421 (2011)
- Kleinberger,G., Capell,A., Brouwers,N., Sleegers,K., **Cruts,M.**, Haass,C., Van Broeckhoven,C.: in vitro studies of the pathogenic effect of misssense mutations on key biological properties of progranulin associated with CNS neurodegeneration. 8th IBRO World Congress of Neuroscience, Florence, Italy, July 14-18 : A398 (2011)

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 on mowat-wilson syndrome

Mutations in the *ZFHX1B* (*SIP1, ZEB2*) gene (chr2q22) cause Mowat-Wilson syndrome (MWS, OMIM 235730) [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 (hypospadias in males), eye defects, and in few patients heart defects (tetralogy of Fallot, septal defects, patent ductus arteriosis, pulmonary arterial sling), cleft palate and sensorineural deafness. Analysis of 220 MWS patients 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 (3 in total) missense mutations affecting the function of a domain of the multi-domain ZFHX1B protein are known [for a recent review, see 4].

Some of our previous work

Our lab was the first to discover Sip1 (Smad-interacting protein-1) by virtue of its binding to the MH2 domain of the BMP effector protein Smad1 [5]. Subsequent work has shown that Sip1 binds to the TGFß/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. Sip1 is a DNA-binding transcription factor (TF) that 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 [6]. Full-length Sip1 binds to the co-repressor CtBP [7] and the chromatin-remodeling co-repressor complex NuRD [8]. It can become an activator as well by binding to P300/PCAF [9]. Sip1's levels are under control of miRNAs, including in epithelial-mesenchymal transition, which is relevant to invasive properties of epithelial-derived tumor cells [10-13].

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 [14-16]. Our published data obtained in *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 [15,16]. Our other studies, addressing Sip1 function in the embryonic and early postnatal CNS, particularly in the cortex, have caught attention in the field of cell fate specification in the brain cortex [17,18]; in particular, we revealed new Sip1-controlled mechanisms emanating from postmitotic neurons in cell fate determination of progenitor cells in the normal embryonic brain cortex. Recently, we documented in a new mouse model the genetic interaction of *Zfhx1b/Sip1* with *Sox10* in the enteric nervous system (ENS), another HD gene [19] and in collaborations with J. Haigh (Gent), using our floxed *Sip1* mice, a crucial role of Sip1 in embryonic hematopoiesis was identified [20].

Work performed in year 1 with the support of the Q.E.M.F.

Our work performed in the context of the Q.E.M.F. funding mainly uses 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 1 of the Q.E.M.F. project are listed below in a separate section. The major focus in the first year was to continue to explain in detail the embryonic origin of major clinical signs of MWS as well as document the newly found defects, via our work with mouse models, suggesting a dual mode of function of Sip1 [Conidi, Cazzola *et al.*, 2011], important roles of Sip1 in nociception and pain [Jeub *et al.*, 2011], and in differentiation of oligodendrocyte precursor cells to myelinating cells [Weng *et al.*, *in press*].

In addition, we have made 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, Seuntjens *et al.*, *in preparation*]. Finally, we have also found a new role for Sip1 in a subgroup of motor neurons in the spinal cord [Roy *et al.*, *submitted, under review*]. 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.*, *in press*).

The progress of other, more recently started Sip1 projects supported by the Q.E.M.F. will be reported in the next report.

1. Sip1 controls pain sensitivity via modulation of DRG neuron excitability, and reduced Sip1 levels cause hypoalgesia

In exciting work in collaboration with the German Mouse Clinic (www.mouseclinic.de) in Munich, where we shipped a large cohort of control and adult *Sip1* heterozygous (+/-) mice (-/- mice are not viable as they die early in embryogenesis), carrying a mutant *Sip1* allele that is conceptually comparable to those in MWS patients, for phenotyping. In some of their assays, including the hot plate and the pathological tail flick assay, we have discovered in these morphologically normal, mutant mice a deficient response towards thermal and mechanical noxious stimuli.

To understand the cause of this hypoalgesia in these mutant mice and trace it back to the embryogenesis phase, we removed both floxed *Sip1* alleles from NCC precursors by using a Wnt1-Cre deleter strain. Doing so, we discovered using extensive marker analysis that Sip1 was essential for the generation of boundary cap stem cells. These stem cells cluster at the entry and exit points of peripheral nerve roots during the initial wave of NCC migration. As such, this transient stem cell compartment constitutes a source of the late migration wave of cells bound to the dorsal root ganglia (DRG) and that differentiate into satellite glial cells and late-born nociceptive neurons, and into Schwann cells that accompany motor axons leaving from the spinal cord, and all these cells are defective in these NCC-specific knockout mouse embryos. These embryological data need further study before publication but have prompted us to document the Sip1-dependent generation during embryogenesis of the primary sensory neurons in the body, i.e. those in the DRG, which are in the adult animal the cells responsible for transducing stimulus energies in peripheral tissues (including pain and, by extension, also chronic pain) and passing the resulting sensory signal on to the CNS. In addition, we could combine this with electrophysiological studies of DRG, using Sip1+/- mice, through collaboration with expert teams of the University of Bonn.

Pain is an important signal alerting the body to imminent or ongoing tissue injury. The perception of pain is initiated by the detection of noxious stimuli by the peripheral endings of primary nociceptive neurons. These are a specialized group of small-diameter pseudounipolar neurons with cell bodies in the DRG, as mentioned above. They give rise to thinly myelinated (Aδ-fibers) or unmyelinated (C-fibers) afferent fibers, which convey the signal to the dorsal horn of the spinal cord. The excitability of nociceptors seems to be crucially important in setting the level of pain sensitivity. Disrupting excitability by genetic deletion of ion channels expressed in nociceptive DRG neurons is known to invariably affect pain sensitivity. In addition, manifold changes in voltage-gated ion channels have been observed in chronic pain syndromes. We found in our studies that Sip1 plays a role in controlling pain sensitivity as mutant mice lacking one *Sip1* allele display reduced thermal pain responses whereas mechanical pain is unaffected. Electrophysiological measurements revealed a reduced spike gain only in capsaicin/heat-sensitive DRG neurons. This was accompanied by an up-regulation of persistent Na^+-channels and a decrease of delayed rectifier K^+-channels. Together with other results these results indicate that Sip1 regulates thermal pain sensitivity by controlling the transduction properties of nociceptive primary sensory neurons in a novel manner via changes in DRG voltage-gated ion channels [Jeub *et al.*, 2011]..

2. Sip1 is part of anti-BMP/anti-Wnt control mechanisms that are crucial for the differentiation of oligodendrocyte precursor cells into myelinating cells

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. We had noted the presence of Sip1 in Schwann cells accompanying the limb motor axons in the mouse embryo (see section 1 above). This prompted them to engage in studies aiming at documenting the possible role of Sip1 as BMP-Smad activity modulator in myelination. The bHLH-type TFs Olig1 and Olig2 are known to promote myelination, whereas BMP and Wnt/ß-catenin signaling inhibit myelination. The data at the start showed that Olig1/2 directly activated *Sip1* in NPCs and that *Sip1* it is a common direct target (as determined by chromatin-immunoprecipitation, ChIP) for these Olig TFs. This made Sip1 a new candidate regulator, downstream of Olig1/2, of the myelinogenic program in the CNS.

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 OGs, 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. Furthermore, Sip1 was found to activate the expression of crucial myelination-promoting factors normally inhibited by Wnt/ß-catenin signaling, while it inhibits the myelination-inhibiting BMP signaling by antagonizing the activity of activated BMP-Smads within the same cells. This supports previous observations by our team that Sip1 can act as repressor and activator of transcription. In addition, 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/ß-catenin negative regulatory pathways. Smad7 overexpression can rescue the differentiation defects of Sip1 mutant OPCs. For this, they will use lentivirus-mediated Smad7 transduction in control and Sip1-knockout cortices and assess the rescue and hence the presence of MBP-positive OGs. In addition, Smad7 is required and sufficient

for negatively regulating Wnt/ß-catenin signaling that inhibits OG myelination. For this, overexpression of Smad7 alone or Smad7 combined with Smurf1 in rat OPCs was used, and the effects on BMPR levels analyzed and, more importantly, the expected decrease in stabilized ß-catenin levels. Indeed, the Smad7-Smurf complex targets and degrades TGFβ/BMP receptors by ubiquitination, thereby attenuating TGFβ/BMP. Smad7 was also reported to negatively regulate Wnt/ß-catenin signaling, while ß-catenin stabilization inhibits OG myelination.

So, our study [Weng *et al.*, *in press*] on Sip1 identified two new candidate mediators for myelin repair, Sip1 and Smad7, which can now be further studied in other mouse models, including those addressing remyelination and/or demyelinating disease. Finally, the critical role of Sip1 in CNS myelination discovered through this work 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 molecular node of the regulatory network that integrates different signaling pathways and transcriptional signals that govern CNS myelinogenesis. In addition, modulation of the Smad signaling pathway may provide a future effective means to promote brain repair in patients with devastating demyelinating diseases and other neurological disorders of the CNS.

3. Sip1 is a novel regulator of visceral motor neuron differentiation in One-cut 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 different embryonic regions, including the spinal cord. In parallel, colleague F. Clotman (UCL, Brussels), in a context of studies in the spinal cord on other TFs of the One-cut class, used our anti-Sip1 antibodies to document its expression in the spinal cord and found that this labels a specific set of motor neurons in the spinal cord. This work revealed yet another function of Sip1 in the CNS Roy *et al.*, *submitted*, *under review*].

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 several transcriptional regulators including the LIM-HD 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 transcriptional activators of the One-cut family critically regulate MN subtype diversification during spinal cord development. One-cut factors do that by directly stimulating *Isl1* expression in specific MN subtypes and are therefore required to maintain Isl1 production at the time of MN diversification. In the absence of One-cut 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. It is this aspect of the study that identified Sip1 as a novel developmental regulator of visceral MN differentiation as well.

More specifically, the absence of Sip1 in prospective visceral MN at E10.5 in *Hnf6/Oc2-/-* embryos suggested that One-cut factors control the onset of *Sip1* reactivation in differentiating MN. However such requirement for OC factors in *Sip1* expression seems to be transient, as Sip1 was present in *Hnf6/Oc2*$^{-/-}$ 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, we studied MN development in our Brn4Cre;Sip1mutant embryos, wherein *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, Sip1 is not required for MN generation and identity consolidation. At E12.5, the somatic MN were present in normal amount and displayed proper columnar organization. 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 neither due to cell death, as no increase in apoptosis was detected in the spinal cord of Brn4Cre;Sip1mutant embryos at E12.5, nor to fate conversion of visceral MN into V2a interneurons, as the amount of Chx10-positive V2a cells was normal. Taken together, these observations demonstrate that the presence of Sip1 between E10.5 and E12.5 is required for the production of proper amounts of visceral MN and seems to have effects opposite to One-cut factors in this process. Therefore, the stimulation of *Sip1* expression by OC factors in newly-born MN might provide an additional feedback mechanism to adjust visceral MN production.

4. Sip1 is essential for GABAergic interneuron migration in the embryonic forebrain

Our team addressed Sip1 function in embryonic and early postnatal CNS, and has caught important attention in the field of cell fate specification in the brain cortex because of the non-cell autonomous role of this TF there [18]. We have extended studies on Sip1 in a 2nd area of the embryonic forebrain where Sip1 is present, i.e. the basal ganglia of the ventral telencephalon (VT), in GABAergic interneurons (INs). These are generated by the ganglionic eminences (GEs), with the media GE generating about 70% of INs in the cortex and the caudal GE 30%. 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) and is likely coordinated by sets of intrinsic and extrinsic factors. These provide cues whose identity may overlap with those known from axon guidance of non-cortical neurons at other sites in the CNS. For example, in the VZ of the MGE Slit proteins and Ephrin receptors (e.g.EphA4/5) act as chemorepellents, and the striatal Semaphorins (Sema3a/3f) inhibit the interneurons from invading this area. A lot of questions on the precise actors and their regulation in migration and guidance of these cortical INs remain unanswered.

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. Using these models for Sip1, *in vivo* and *in vitro* analyses and transcriptomics (using Sip1-deficient, Cre-activated GFP-positive FACS-sorted forebrain cells, followed by RNA-Seq analysis by the Grosveld team, Rotterdam), 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 (the majority of which are upregulated in the absence of Sip1) and not for their migratory capacity *per se*. More work is needed, including investigation of cell-cell (de)adhesion and/or intrinsic migration. Furthermore, the work also revealed a relation to seizures and epilepsy observed in the majority of MWS patients.

The major urgent objective is 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. For this, the our team has already set up focal electroporation (FEP) in organotypic brain slices made from E13.5 wild-type, $Sip1^{flex7/flex7}$ and Sip1 knockout embryos. These slices can be injected/electroporated with vectors either encoding a shRNA or cDNA. After FEP, slices are cultured for up to 3 days and subsequently analyzed

via confocal microscopy, and the migration of the traceable cells quantified. Based on preliminary data, where it was tried to mimic the *Sip1* mutant phenotype by overproduction of Netrin1 and Unc5b (both upregulated in the mutant brains) in wild-type MGEs via FEP, they will now go systematically through the list of candidate cues, including those of the Netrin/Netrin receptor family.

5. The emerging dual face of Sip1 as transcriptional activator and repressor

A number of important general conclusions can be drawn from our studies with previous and presently studied conditional *Sip1* knockout mice. First, some of the phenotypes found in the respective knockout mice correlate with defects found in MWS patients, but other ones reveal new roles of Sip1 in certain cell types/tissues, which have not been analyzed yet in patients. For example, our studies are revealing important new functions of Sip1 in the adrenosympathic anlage and in the epicardial cells during cardiac development (not shown), in myelination and in pain perception, and in GABAergic interneuron biology relating to seizures and epilepsy. Indeed, selective removal of Sip1 from GABAergic interneurons in the ventral forebrain, at least with some of the used Cre strains in our ongoing studies, yield mice that three weeks after birth undergo myoclonic seizures and die immediately after. Second, in many cases the established conditional mouse models display phenotypes the molecular mechanisms of which reveal also new modes of action of Sip1. For example, a number of genes that help to explain the phenotype(s) are downregulated in the Sip1 knockout cells, while many more other genes are upregulated in the absence of Sip1, pointing at Sip1 as being an activator and for the majority of its target genes a repressor of target gene transcription. Many of these genes are candidate direct target genes for Sip1 and/or point also at other cellular processes where Sip1 could play a role. For example, RNA-seq analysis of sorted Sip1-deficient embryonic forebrain cells, and comparison with sequencing data from control forebrains, suggest regulation of different classes of genes involved not only in neurogenesis but also the regulation of gene sets encoding GPCRs and ion channels, vesicular trafficking proteins, and proteins involved in synaptogenesis and synaptic plasticity.

Third, the picture is emerging that Sip1 negatively regulates BMP-Smad signaling in a number of multipotent progenitor cell types where BMPs exert an anti-differentiation effect, e.g. anti-neural effects of BMPs in Xenopus embryos and mouse embryonic stem cells (studies ongoing), while Sip1 is necessary for neuroectodermal differentiation of human ES cells [21], but also for embryonic haematopoiesis [20] and myelination [our paper Weng *et al.*, *in press*]. This means that evidence is accumulating that Sip1 is an intracellular negative regulatory mechanism of BMP-Smad signaling in the nucleus of ligand-activated cells by virtue of binding to the R-Smads, and where the candidate target genes for the Smad-Sip1 repressive interaction are genes that are otherwise BMP-induced and encode negative regulators of cell commitment/differentiation. Following the same logic, it would in the same cells also be very well possible that Sip1 as a transcriptional activator then directly activates a set of genes that promotes the differentiation process. Fourth, it cannot be excluded that Sip1 has in addition to its cell-autonomous role also a non-cell autonomous function and hence in the knockout models its removal from a specific subset of cells has also consequences for other cells in the same region or niche when Sip1 is not expressed in these latter cells. This is clearly the case in the embryonic cortex in the forebrain, where Sip1 in neurons of the upper layers regulates the level of transcripts for the secreted proteins neurotrophin-3 and fibroblast growth factor-9, which regulate the timing of neurogenesis and gliogenesis, respectively, of the progenitor cells [18]. These findings in studies with Sip1 were therefore also discussed in a published manuscript, in a broader context of TGFß/BMP family Smad signalling [Conidi, Cazzola *et al.*, 2011].

Manuscripts with support from the q.e.m.f.

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

- Dec. 16, 2011: DH: *Seminar* at the College of Medicine, Biological Sciences and Psychology, Cancer Theme / Molecular and Cellular Bioscience, University of Leicester, UK (Dr. E. Tulchinsky)
- Nov. 23, 2011: ES: *Seminar* at the University of Lleida, Institute of Biomedical Research, Spain (Dr. J. Egea)
- Sept. 29, 2011: DH: *Seminar* at the Max-Planck-Institute for Heart and Lung Research (T. Braun), Bad Nauheim, Germany, in the context of the Excellence Cluster Cardio Pulmonary System (ECCPS) symposium (T. Braun, W. Seeger, A. Zeiher, coords)
- Sept. 15, 2011: DH: Invited keynote speaker at the 21st MGC-Medical Genetics Center Symposium, Univ. Leiden, The Netherlands (orgs. M. Nivard, F. Grosveld)
- July 1-2, 2011: ES: Selected speaker at the Belgian Society for Cell and Developmental Biology (BSCDB) meeting on Neural Specification and Patterning in the Embryo (E. Bellefroid, org), Rochehaut, Belgium
- June 30, 2011: DH: *Seminar* at the Nijmegen Centre for Molecular Life Sciences (NCMLS), Radboud University Nijmegen, The Netherlands (Dr. J. van Zoelen)
- June 23, 2011: DH: *Seminar* at the Cancer Genomics & Developmental Biology program of the Utrecht University and the Hubrecht Institute, Utrecht, The Netherlands (Dr. H. Bos, Dr. B. Burgering)
- May 20, 2011: DH: *Seminar* at Leiden University Medical Center, Laboratory for signal transduction mechanisms of TGFß (Dr. P. ten Dijke)
- Mar.15, 2011: DH: *Seminar* at Dept Biological Sciences and Biotechnology, Tsinghua University, Beijing, China (Dr. Y-G. Chen)
- Feb-June, 2011: DH: 5 lectures on TGFß signalling, within the framework of the awarded visiting professorship at dept. Cell Biology (director: Dr. F. Grosveld), Erasmus MC, Rotterdam, The Netherlands (Feb. 22, Mar. 9, May 11, June 7, June 21)
- Jan. 25, 2011: DH: *Seminar* at the Luxembourg Centre for Systems Biomedicine (LCSB), Luxembourg (Dr. R. Balling)

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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 the organization of the experiments detailed in the research proposal, namely the characterization by functional magnetic resonance imaging of the influence of local sleep need and circadian signals on human regional brain function. Data acquisition is underway and the analyses should begin during spring 2012. We are currently designing the same experiment using TMS/EEG in order to investigate effective brain connectivity during sleep loss.

In consequence, this year, our publications essentially deal with our previous research projects, aiming at characterizing spontaneous brain activity and its influence on brain processing.

2. Characterization of spontaneous brain activity during normal human sleep and anesthesia

2.1 Brain functional integration decreases during propofol-induced loss of consciousness

Consciousness has been related to the amount of integrated information that the brain is able to generate. In this paper, we tested the hypothesis that the loss of consciousness caused by propofol anesthesia is associated with a significant reduction in the capacity of the brain to integrate information. To assess the functional structure of the whole brain, functional integration and partial correlations were computed from fMRI data acquired from 18 healthy volunteers during resting wakefulness and propofol-induced deep sedation. Total integration was significantly reduced from wakefulness to deep sedation in the whole brain as well as within and between its constituent networks (or systems). Integration was systematically reduced within each system (i.e., brain or networks), as well as between networks. However, the ventral attentional network maintained interactions with most other networks during deep sedation. Partial correlations further suggested that functional connectivity was particularly affected between parietal areas and frontal or temporal regions during deep sedation. Our findings suggest that the breakdown in brain integration is the neural correlate of the loss of consciousness induced by propofol. They stress the important role played by parietal and frontal areas in the generation of consciousness.

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2.2 Hierarchical clustering of brain activity during human non rapid eye movement sleep

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 summarized in a publication which is submitted for publication.

2.3 Interplay between spontaneous and induced brain activity during normal human sleep

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

Dang-Vu TT, Bonjean M, Schabus M, Boly M, Darsaud A, Desseilles M, Degueldre C, Balteau E, Phillips C, Luxen A, Sejnowski TJ, Maquet P (2011) Interplay between spontaneous and induced brain activity during human non-rapid eye movement sleep. Proc Natl Acad Sci U S A 108:15438-15443.

2.4 Experience-dependent induction of hypnagogic images during daytime naps: a combined behavioral and EEG study.

This study characterizes hypnagogic hallucinations reported during a polygraphically-recorded 90-minute daytime nap following or preceding practice of the computer game Tetris. In the experimental group (n=16), participants played Tetris in the morning for two hours during three consecutive days, while in a first control group (n=14, controlling the effect of anticipation), participants played Tetris after the nap and in a second control group (n=13, controlling the effect of experience), participants did not play any game. During afternoon naps, participants were repetitively awakened 15, 45, 75, 120 or 180 seconds after the onset of sleep stage 1 and were asked to report their mental content. Reports content was scored by three judges (inter-rater reliability 85%). In the experimental group, 48 out of 485 (10%) sleep-onset reports were Tetris-related. They mostly consisted of images and sounds with very little emotional content. They exactly reproduced Tetris elements or mixed them with other mnemonic components. By contrast, in the first control group, only 3 reports out of 112 were scored as Tetris-related and in the second control group only 1 report out of 107 (1%) (between-groups comparison; p=0.006). Hypnagogic hallucinations were more consistently induced by experience than by anticipation (p=0.039) and they were predominantly observed during the transition of wakefulness to sleep. The observed attributes of experience-related hypnagogic hallucinations are consistent with the particular organization of regional brain activity at sleep onset, characterized by high activity in sensory cortices and in the default mode network.

Kusse C, Shaffii Lebourdiec A, Schrouff J, Matarazzo L, Maquet P (2011) Experience-dependent induction of hypnagogic images during daytime naps: a combined behavioural and EEG study. J Sleep Res.(ahead of print)

2.5 Neural correlates of ongoing conscious experience: both task-unrelatedness and stimulus-independence are related to default network activity

The default mode network (DMN) is a set of brain regions that consistently shows higher activity at rest compared to tasks requiring sustained focused attention toward externally presented stimuli. The cognitive processes that the DMN possibly underlies remain a matter of debate. It has alternately been proposed that DMN activity reflects unfocused attention toward external stimuli or the occurrence of internally generated thoughts. The present study aimed at clarifying this issue by investigating the neural correlates of the various kinds of conscious experiences that can occur during task performance. Four classes of conscious experiences (i.e., being fully focused on the task, distractions by irrelevant sensations/perceptions, interfering thoughts related to the appraisal of the task, and mind-wandering) that varied along two dimensions ("task-relatedness" and "stimulus-dependency") were sampled using thought-probes while the participants performed a go/no-go task. Analyses performed on the intervals preceding each probe according to the reported subjective experience revealed that both dimensions are relevant to explain activity in several regions of the DMN, namely the medial prefrontal cortex, posterior cingulate cortex/precuneus, and posterior inferior parietal lobe. Notably, an additive effect of the two dimensions was demonstrated for midline DMN regions. On the other hand, lateral temporal regions (also part of the DMN) were specifically related to stimulus-independent reports. These results suggest that midline DMN regions underlie cognitive processes that are active during both internal thoughts and external unfocused attention. They also strengthen the view that the DMN can be fractionated into different subcomponents and reveal the necessity to consider both the stimulus-dependent and the task-related dimensions of conscious experiences when studying the possible functional roles of the DMN.

Stawarczyk D, Majerus S, Maquet P, D'Argembeau A Neural correlates of ongoing conscious experience: both task-unrelatedness and stimulus-independence are related to default network activity. PLoS One 6:e16997.

3 Light and the regulation of sleep and wakefulness

Light is a powerful modulator of cognition through its long-term effects on circadian rhythmicity and direct effects on brain function as identified by neuroimaging. How the direct impact of light on brain function varies with wavelength of light, circadian phase, and sleep homeostasis, and how this differs between individuals, is a largely unexplored area. Using functional MRI, we compared the effects of 1 minute of low-intensity blue (473 nm) and green light (527 nm) exposures on brain responses to an auditory working memory task while varying circadian phase and status of the sleep homeostat. Data were collected in 27 subjects genotyped for the PER3 VNTR (12 PER3(5/5) and 15 PER3(4/4)) in whom it was previously shown that the brain responses to this task, when conducted in darkness, depend on circadian phase, sleep homeostasis, and genotype. In the morning after sleep, blue light, relative to green light, increased brain responses primarily in the ventrolateral and dorsolateral prefrontal cortex and in the intraparietal sulcus, but only in PER3(4/4) individuals. By contrast, in the morning after sleep loss, blue light increased brain responses in a left thalamofrontoparietal circuit to a larger extent than green light, and only so in PER3(5/5) individuals. In the evening wake maintenance zone following a normal waking day, no differential effect of 1 minute of blue versus green light was observed in either genotype.

Comparison of the current results with the findings observed in darkness indicates that light acts as an activating agent particularly under those circumstances in which and in those individuals in whom brain function is jeopardized by an adverse circadian phase and high homeostatic sleep pressure.

Vandewalle G, Archer SN, Wuillaume C, Balteau E, Degueldre C, Luxen A, Dijk DJ, Maquet P (2011) Effects of light on cognitive brain responses depend on circadian phase and sleep homeostasis. J Biol Rhythms 26:249-259.

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 α-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. 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 radial migration and the control of the interkinetic nuclear movement (INM). These biological events have previously been linked to connexin expression but not its acetylation. We

are currently identifying the lysine residues 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. Finally, will performed time-lapse recordings to study radial migration as well as the INM kinetics.

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. 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. 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. Altogether, these results indicate that Elp3 seems to be involved in neuronal survival and axonal guidance in the cochlea.

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- Beukelaers, P., Vandenbosch, R., Caron, N., **Nguyen, L.**, Belachew, S., Moonen, G., Kiyokawa, H., Barbacid, M., Santamaria, D., and **Malgrange, B.** : Cdk6-Dependent Regulation of G(1) Length Controls Adult Neurogenesis.
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  Cell Mol Life Sci (2011) (I.F. 2010= 7.047)

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– Westerlund, N., Zdrojewska, J., Padzik, A., Komulainen, E., Björkblom, B., Rannikko, E., Tararuk, T., Garcia-Frigola, C., Sandholm, J., **Nguyen, L.**, Kallunki, T., Courtney, M.J., and Coffey, E. [CA] : SCG10/Stathmin phosphorylation determines the duration of multipolar stage and migration speed.
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Cell Mol Life Sci (2011), 68(4): 635-49 (I.F. 2010= 7.047)

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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 2011 thanks to the support from FMRE/GSKE is summarized below.

1. Deciphering the role of D1R-striatonigral and D2R-striatopallidal MSN of striatal subregions in motor control, instrumental learning and drug addiction.

1.a Effects of specific ablations D1R-striatonigral and D2R-striatopallidal MSN in distinct dorsal striatum subregions.

Using a transgenic A2AR-Cre mouse strain that we developed (Durieux et al., 2009,2011a) 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 D2R-MSN can be performed in different restricted functional areas of the striatum and produce specific behavioural alterations. Indeed, D2R 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 D2R striatopallidal MSN exert inhibitory functions on both locomotor control and drug reward process (Durieux et al., 2009,2011a). We have now developed a parallel model allowing specific removal of the D1R striatonigral MSN (Durieux et al., 2011b) 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 D2R and D1R MSN exert dissociated control over motor control and motor skill learning. Indeed, full ablation of these D1R striatonigral MSN led to a profound and persistent reduction in locomotion (by 31%) (Durieux et al., 2011b) while, as demonstrated in our previous papers (Durieux et al., 2009,2011a), mice with a specific ablation of D2R striatopallidal MSN exhibited persistent hyperactivity (about 400%) (Durieux et al., 2011b). These results provide direct experimental evidence for an opposite control of the two populations over motor activity in freely moving animals, showing that D2R- and D1R-MSNs inhibit and stimulate motor activity respectively. The dorsal striatum is not only involved in the planning of new motor tasks but also in motor learning. Motor skill learning requires repetitive training during which performance typically shows initial fast improvements,

followed by slower ameliorations to reach a plateau that represent progressive skill automatisation (Luft and Buitrago, 2005). To assess for the respective roles of D1R striatonigral and D2R striatopallidal MSN in this process, mice were trained in a motor skill learning task on an accelerating rotarod. In this task, mice have to learn a novel sequence of movements to maintain balance on a rotating rod in constant acceleration and receive several trials per day for consecutive days. Full ablation of D2R striatopallidal MSN resulted in early impairments in the rotarod task, with a progressive improvement of performance that finally reach control level. In contrast, mice with full ablation of D1R striatonigral MSN were unable to learn the task and displayed a permanent deficit (Durieux et al., 2011b). In view of these cell-type specific deficits during the rotarod acquisition, we investigated impact of D1R- or D2R-MSN removal after extensive rotarod training. While D1R-MSN ablated mice displayed profound rotarod impairments, mice lacking D2R-MSN showed similar performances as compared to controls. This showed that execution of a previously learned motor sequence is not dependent on the D_2R-MSN pathway while D1R-neurons are still necessary for performance (Durieux et al., 2011b).

Furthermore, the dorsal striatum is subdivided into an external portion (the dorsolateral striatum, DLS, corresponding to the primate putamen, predominantly innervated by the sensorimotor cortex) and an internal part (the dorsomedial striatum, DMS, homologous to primate caudate nucleus, receiving projections from prefrontal and other association cortices) (Graybiel, 2008). While the DMS seems 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), the DLS seems required for progressive skill automatization and habit learning (Yin et al., 2004, 2009). However, again, the specific involvement in motor learning phases of striatopallidal and striatonigral neurons in the different striatal sectors was completely unknown.

We have now successfully designed new protocols allowing to ablate D1R striatonigral and D2R striatopallidal MSN restrictedly in the DLS and DMS using the same transgenic lines (Durieux et al., 2011b). Elimination of D1R-striatonigral neurons in the DMS induced a reduction in ambulation that was not observed following the DLS lesion. In contrast, DMS D2R-striatopallidal neuron ablated mice displayed hyperlocomotion while D2R-striatopallidal neuron loss in the DLS did not produce any locomotor activity increase. This showed that the modulatory influence on locomotion observed following full ablations was partially recapitulated in DMS, but not in DLS, restricted ablations, indicating that associative striatum area exerts a MSN population-dependent control over spontaneous locomotion. Since we also noted impairment in novel environment habituation, we tested whether or not novel object exploration and recognition were also altered by using a decoupled delayed spontaneous object recognition task (McTighe et al., 2010). This showed that, as for locomotor activity, DMS specific ablations, but not the DLS lesions, demonstrate a cell-type specific modulation of novel object exploration in which D2R-MSNs and D1R-MSNs inhibit and stimulate novelty exploration, respectively (Durieux et al., 2011b). This indicate that the direct pathway in the associative cortico-striatal loop (DMS) is necessary for novelty-induced exploration and that in DMS D2R-neuron ablated mice, a continuous translation of sensory stimuli to locomotion would lead to a state of continuous exploration/locomotion.

Evaluation of involvement of DLS and DMS D1R striatonigral and D2R striatopallidal neurons in motor skill learning in the accelerating rotarod task showed that DMS D2R-neuron ablated mice were impaired during initial trials but gradually improved their performances to reach control levels while D2R-neuron elimination in the DLS did not affect the task. On the other hand, mice lacking D1R-expressing neurons in the DLS showed profound rotarod impairments, an effect totally absent in DMS D1R-neuron ablated mice (Durieux et al., 2011b). These results suggest that in naive animals, when task performance is more susceptible to interference and more dependent on attention (Luft and Buitrago, 2005), D1R- and D2R-MSNs work in concert to promote acquisition of a new motor skill: activation of D1R-MSNs in the sensorimotor striatum is required for progressive automaticity of task performance, by development of

correct motor strategies (Graybiel, 2008), while activation of D2R-MSNs in associative striatum inhibits competing exploratory activity. During later skill learning stage, attention to action is less required and DMS D2R-MSNs progressively disengage of the process while DLS D1R-MSN pathway is still required for skill automatization.

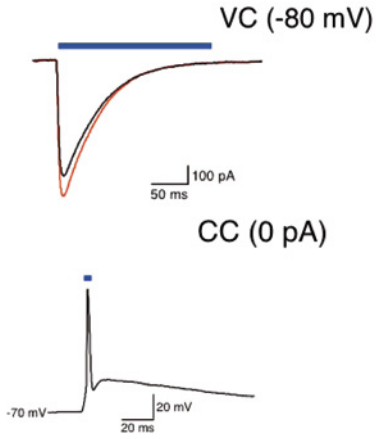
Striatal neurons are the main direct or indirect targets of pharmacological treatments in movement disorders as Parkinson's disease or in schizophrenia as well as of drugs of abuse but the involvement of D1R striatonigral and D2R striatopallidal MSN in the DMS and DLS, respectively, remained unknown. For instance, treatment of schizophrenia positive symptoms with typical neuroleptic drugs is often associated with motor side effects such as catalepsy. We then evaluated involvement of each neuronal population in motor responses to neuroleptic drugs. This revealed that D2R-striatopallidal neuron removal selectively in the associative striatum completely abolished haloperidol–induced immobility and catalepsy. Neither ablation of D2R-neurons in the DLS nor D1R-neuron ablations in both dorsal striatum subregions altered haloperidol responses, indicating that, in normal subject, D2R-antagonism in striatopallidal neurons of the associative striatum (DMS) is critical for the motor effects of haloperidol (Durieux et al., 2011b).

Behavioural sensitization as locomotor sensitization to psychostimulants is produced by repeated drug administration and is defined as an increase in the locomotor effect of the drug upon re-administration. We evaluated involvement of each neuronal population in these motor responses to psychostimulant. Acute amphetamine locomotor response was increased in mice lacking DLS striatopallidal neurons while DMS D2R-neuron ablated mice displayed a deficit in amphetamine response sensitization, suggesting that sensorimotor and associative striatum indirect pathways are normally involved in regulation of acute amphetamine locomotor response and its sensitization respectively. In contrast, dorsal striatum D1R-neuron ablations did not affect amphetamine sensitization, but DMS direct pathway neuron removal reduces acute locomotor response to amphetamine. The interpretation of amphetamine acute effects is not straightforward due to the effects of region-selective neuron ablations on spontaneous locomotion. However, although numerous studies supported a specific role of the ventral striatum as the main striatal region contributing to the development of psychostimulant sensitization (Steketee and Kalivas 2011), our results strongly point to a necessary contribution of DMS D2R-neurons in amphetamine sensitization (Durieux et al., 2011b).

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 D1R-striatonigral and D2R-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 plan 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 started by 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., 2009,2011a). 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 (VC) and in the evocation of action potentials when recorded in current clamp (CC) . Establishment of the design for *in vivo* behavioural paradigms is running.

1.c Specific inactivation of NR1 in striatopallidal 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 D2R striatopallidal MSN (see above and Durieux et al., 2009,2011a), 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 we are now crossing his NR1 floxed mice with our A_{2A}R-Cre/+ mice to obtain new A_{2A}R-Cre/+ NR1$^{f/f}$. This will allow to obtain a better cell-specific NR1 inactivation in both D1R striatonigral and D2R striatopallidal neurons and hence to perform a deeper behavioural analysis including instrumental learning and conditioned place preference. 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 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 genes as RGS5, GuaCy13A, Adk that exhibit both a high differential expression and a putative physiological relevance for further analysis using different knock down strategies. Different

knock-out or knock-down strategies have been initiated, from analysis of global knock-out mice when available as for RGS5 knock-out mice, to the development of striatal or striatopallidal neuron selective knock-down using lentivirus-mediated small hairpin RNA for other genes when transgenic mice are not available (Ena et al., 2011). These approaches gave rise to data showing slight alterations in motor skill learning tested on a accelerated rotarod in RGS5 KO mice and substantial alterations in this task in mice knock-down for another striatopallidal MSN-specific gene (Ena et al. in preparation).

2. Regulation of striatal neurons excitability and corticostriatal synaptic transmission

Striatal neurons (MSN) excitability and corticostriatal synaptic transmission are tightly regulated both by a large series of neurotransmitters and by striatal interneurons.

2.a Neuronal excitability of striatal fast-spiking interneurons deficient in parvalbumin

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 by their implication in severe disorders affecting the basal ganglia such as Parkinsonism, dystonia and Tourette syndrome. FSI are the only striatal neurons to express the calcium-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 FSI dendritic integration, spiking mode and neurotransmission, hence their involvement on the striatal microcircuit. 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 linking Ca^{2+} buffering and spiking due to the activation of small conductance (SK) Ca^{2+}-dependent K^+ channels. Furthermore, PV deletion modified short-term plasticity at both, inhibitory FSI to MSN and excitatory cortex to FSI synapses at specific frequencies. PV-deficiency also led to a remodeling of the FSI dendritic tree (Orduz et al., submitted). Our results support the hypothesis that in FSI, PV is crucial for the fine-tuning of the temporal responses of the FSI network and for the synchronous 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 demonstrated in a transgenic model of spinocerebellar ataxia type 1 (SCA1) that neuronal dysfunction contributes to neurodegeneration of cerebellar Purkinje cells (Hourez et al., 2011). Indeed, we demonstrated that an early functional alteration in Purkinje cells resulting from an increase in A-type K+ current (IK_A) causes motor dysfunction before the appearance of any atrophy or neuronal death. In addition, our results also showed that restoring a normal functional behaviour of these neurons by chronic pharmacological treatment with the IK_A blockers aminopyridines, led not only to the restoration of a normal motor activity, but also, unexpectedly, to a slow down of the Purkinje cell degenerative process (Hourez et al., 2011). These data demonstrate that aminopyridines might have symptomatic and neuroprotective beneficial effects in SCA1 and that treatment of early neuronal dysfunction is relevant in neurodegenerative disorders.

- We 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).

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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, its comorbidities and the antiepileptic drugs (AEDs)

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 (1). Cognitive impairment and major depression are common disabilities associated with refractory epilepsy. Important contributing factors may be the overlap in synaptic plasticity mechanisms underlying both memory formation and epileptogenesis (2) as well as abnormalities in transmitter systems (e.g. monoamines) involved in depression and epilepsy (3,4). 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 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).

These clinically used drugs have actually a narrow spectrum of mechanisms of action. Most act by reinforcing the brain's GABA-ergic inhibition or by blocking voltage-gated sodium channels. A few next-generation AEDs interact with novel targets, e.g. gabapentin and pregabalin bind to the $\alpha 2\delta$-1 subunit of voltage-gated calcium channels identified as a neuronal thrombospondin receptor (5) and levetiracetam binds the synaptic vesicle protein SV2A (6). Although these AEDs have a favourable side-effect profile, they have not yet spectacularly reduced the total number of refractory patients. With exception of tiagabine (a GABA reuptake inhibitor) and vigabatrin (a GABA transaminase inhibitor), the main mechanisms of action of all marketed AEDs are situated at the level of the nerve cells. The same holds true for at least eight out of twelve new AEDs in development (7). Again half of these AEDs in the pipeline act on specific voltage-gated ion channels.

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) or halt its progression (disease-modification).

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 composed of a heavy chain subunit common to amino acid transporters, 4F2, and a light chain specific subunit, xCT (8). In the adult brain system x_c^- is restricted to glia (9,10) and exchanges an intracellular glutamate for an extracellular cystine molecule (11). Cystine is intracellularly reduced to cysteine, the rate-limiting substrate in the synthesis of glutathione, the major antioxidant of the brain. In rodent nucleus accumbens but not prefrontal cortex, glutamate release via system x_c^- is the major source of extracellular glutamate (12,13).

It remained elusive whether system x_c^- controls extracellular glutamate in hippocampus and whether system x_c^- is involved in hippocampus-related functions such as spatial learning and memory or pathological conditions such as limbic seizures.

System x_c^- has been extensively studied in brain regions related to drug addiction and is downregulated in nucleus accumbens after chronic drug abuse. Stimulating cystine/glutamate exchange with N-acetylcysteine prevented the reinstatement of drug seeking in rats and diminished the desire for cocaine and cigarettes use in humans (14,15). Although it has been amply suggested that system x_c^- might be an interesting target for the development of new CNS therapies, only a few reports have appeared to date demonstrating its involvement in Alzheimer's disease (16,17,18), Parkinson's disease (19, 20) and amyotrophic lateral sclerosis/Parkinson dementia complex on Guam (21).

I.3. System x_c^- in oxidative stress and glutamate excitotoxicity in epilepsy: a double-edged sword

Oxidative stress results from an imbalance in oxidant and antioxidant homeostasis in favour of the production of reactive oxygen species. Mitochondrial oxidative stress actively contributes to seizures and epileptogenesis (22). Interestingly, the AEDs zonisamide and levetiracetam possess antioxidant and neuroprotective properties (23,24). Levetiracetam altered the hippocampal expression of xCT (25), suggesting a link between an AED mechanism of action and system x_c^-. In the hippocampus of 10 week old EL mice, a genetic model of secondarily generalised seizures, both EAAC1, a neuronal glutamate and cysteine (re)uptake transporter, and xCT expression levels were decreased compared to DDY control mice (26). The authors suggested that depletion of endogenous antioxidant ability due to decreased xCT expression might provide the molecular mechanism to trigger ictogenesis (26).

Unbalanced excitatory-inhibitory transmitter interactions in seizure-prone areas can evoke epilepsy. Enhanced hippocampal glutamate-mediated excitatory action and subsequent seizures can originate from excessive glutamate release, overactivation of glutamate receptors and/or glutamate transporter defects. The applicants have built up ample experience in search for strategies that suppress excessive glutamate receptor-mediated processes in epilepsy (27,28). Although in the current project, glutamate remains a familiar key player, the novelty lies in the shift to a glial transporter target of which the role in hippocampal functioning and seizure mechanisms remains unexplored.

Since system x_c^- will transport cystine into the cell in exchange for an intracellular glutamate molecule, its implication in the pathogenesis of epilepsy can be dual. An increased activity of the antiporter will result in an increased intracellular cysteine store, allowing a higher synthesis rate of glutathione and increased antioxidant capacity, and thus beneficial effects. However, increased system x_c^- activity will also lead to increased extracellular glutamate levels, and thus possibly to proconvulsant effects. The latter situation is indirectly supported by two case reports in which status epilepticus was observed following N-acetylcysteine (a known activator of system x_c^-) therapy (29,30). The current research project will clarify which mechanism is the most important in both physiological and pathological conditions. It will be necessary to study concomitantly the involvement of other players that can affect the net extracellular glutamate levels, such as vesicular glutamate transporters and glial high-affinity Na^+/K^+-dependent glutamate transporters.

II. Aim and objectives of the project

II.1. Recent breakthrough, general aim and specific objectives of the project

We recently started to study the contribution of both oxidative stress and glutamate excitotoxicity in hippocampal physiology by using transgenic mice that lack xCT (xCT knockout or $xCT^{-/-}$ mice), the specific subunit of the antiporter. Although it was suggested that of the various routes for astrocytic glutamate release, perhaps the most evidence is for Ca^{2+}-dependent exocytosis (31), we discovered that system x_c^- is the major source of extracellular glutamate in the hippocampus. Moreover, we observed that the $xCT^{-/-}$ mice are less prone to acute limbic seizures. This breakthrough is the fundament to unveil the role and mechanisms of action of system x_c^- in hippocampus-related (patho)physiological functions.

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 (1), 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.

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. In this respect, we discovered in

a former project supported by GSKE (2008-2010) that the neuropeptide angiotensin IV improved working memory formation (32) and was anticonvulsive against pilocarpine-induced limbic seizures (33). 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.

II.2. Potential impact and multidisciplinarity of the proposal

The benefits of epilepsy research have always extended beyond epilepsy treatment alone. Many AEDs have proven beneficial effects in conditions other than epilepsy, e.g. neuropathic pain, bipolar disorder, migraine, tremor and anxiety. The results of the current project proposal will elucidate the role of system x_c^- in mechanisms of seizures and epilepsy as well as in comorbid disorders such as cognitive decline and depression. These challenging experiments in healthy and diseased rodents and transgenic mice are epoch-making. Maybe a new avenue for the drug treatment of epilepsy will be unveiled.

Moreover, the discovery of a specific, non-transportable inhibitor of system x_c^- will be useful as a potential drug therapy of Alzheimer's disease (16) and of various cancers (34). Indeed, cellular cystine uptake via system x_c^- plays a key role in maintaining glutathione levels in malignant cells as well, and hence preserving their health. Specific inhibition of system x_c^- can result in inhibited growth and reduced drug resistance to chemotherapy of a variety of cancers and cancer cell metastasis (34). Moreover, we recently demonstrated increased expression levels of xCT in the brain of rodent models for Alzheimer's and Parkinson's disease (18, 19) and we showed that mice lacking functional system x_c^- are significantly protected against 6-hydroxydopamine-induced neurodegeneration (model for Parkinson's disease, 20).

III. Results obtained in 2011

In 2011, we made substantial progress in specific items described in work packages 3, 4 and 5, as will be explained in more detail below. These data were compiled into one manuscript that has been accepted by the prestigious Journal of Neuroscience.
Note: We also finished 2 studies in the frame of the previous project supported by GSKE, this has led to another 2 manuscripts accepted in Seizure - European Journal of Epilepsy and Brain Research.

III.1. Progress made in work package 3, brain morphology, extracellular glutamate levels and oxidative stress markers in hippocampus

System x_c^- exchanges intracellular glutamate for extracellular cystine, giving it a potential role in intracellular glutathione (GSH) synthesis and non-vesicular glutamate release.

Hippocampal glutathione (GSH) content and oxidative stress-related markers in xCT$^{-/-}$ mice
xCT deletion in young (12-16 weeks) or old (12-18 months) mice did not affect the hippocampal expression pattern of several oxidative stress-related markers, such as nitrotyrosine, 4-hydroxy-2-nonenal and heme oxygenase-1. For hippocampal GSH content, we observed an age-dependent increase but no effect of genotype. These observations suggest that xCT$^{-/-}$ mice can compensate for the loss of cystine import through system x_c^- and subsequent shortage of cysteine for GSH synthesis.

Lack of brain atrophy and glial cell loss in the hippocampus of xCT$^{-/-}$ mice
No genotype differences were observed between xCT$^{-/-}$ and xCT$^{+/+}$ mice for the surface areas of the dorsal hippocampus over its total rostrocaudal axis in either young mice or old mice. We did not observe

an effect of genotype but observed an overall effect of age on cortex width. No genotype differences or age differences were observed in young or old mice for the width of the CA1 region. Similarly, no genotype differences or age differences were observed in young or old mice for the width of the pyramidal cell layer in the CA1. This indicates the absence of extensive neurodegeneration. Moreover, no genotype effects were observed for the number of GFAP-positive cells per area in the CA1, CA3 and dentate gyrus (DG) of the hippocampus in young and old $xCT^{-/-}$ and $xCT^{+/+}$ mice. However, an increase in the number of GFAP-positive cells per area was observed in the CA1, CA3 and DG in old compared to young mice.

No changes in hippocampal glutamate transporter expression in $xCT^{-/-}$ mice

We also studied possible compensatory changes in hippocampal protein expression of the major glial glutamate transporters GLT-1 and GLAST, the neuronal EAAC1 transporter that can also function as a cysteine transporter, and the vesicular glutamate transporters (VGLUT1-3) in response to xCT gene deletion. Hippocampal expression levels of the vesicular glutamate transporters or synaptophysine, a synaptic vesicle protein whose abundance provides a synaptic marker, were not different between $xCT^{-/-}$ and $xCT^{+/+}$ mice. Hippocampal expression of VGLUT1 and 3 as well as synaptophysine showed an age-dependent decrease, contrary to VGLUT2. Also, the expression levels of the glial high-affinity Na^+/K^+-dependent glutamate transporters (GLT-1 and GLAST) and the neuronal EAAC1 transporter were not different between $xCT^{-/-}$ mice and $xCT^{+/+}$ littermates. Though, expression levels of GLAST, GLT-1 as well as EAAC1 were decreased in aged mice, independent of genotype. In conclusion, these findings demonstrate that, although as a result of ageing, all transporters related to glutamate reuptake as well as two vesicular glutamate transporters are downregulated in hippocampus, protein expression levels of all glutamate transporters are unaffected by genotype and that no compensatory up- or down-regulations are observed due to the loss of xCT protein.

System x_c^- is an important source of extracellular glutamate in the mouse hippocampus

We observed a significant effect of genotype on glutamate dialysate concentrations with significantly lower glutamate concentrations in baseline dialysis samples obtained from young as well as old $xCT^{-/-}$ mice compared to their age-matched $xCT^{+/+}$ littermates. We measured in the same dialysates the levels of aspartate, which is not a substrate for system x_c^-, as a negative control. No difference in extracellular aspartate levels could be observed between $xCT^{+/+}$ mice and $xCT^{-/-}$ mice, independent of age. Using the ultraslow flow method, we also measured significantly lower real extracellular glutamate concentrations in the $xCT^{-/-}$ mice compared to their wildtype controls. These differences in extracellular glutamate levels cannot be linked to compensatory mechanisms as we showed that the expression levels of none of the glutamate transporters, which are the major determinants of extracellular glutamate, are affected by the loss of xCT.

Inhibitors of system x_c^- decrease extracellular glutamate in rat hippocampus

Rat hippocampal glutamate dialysate levels following pharmacological manipulation were also studied. Lowering Ca^{2+} in the perfusion fluid from 2.3 mM to 1.0 mM did not alter extracellular glutamate levels. Reversal of day-night cycle and performing microdialysis during the dark phase - when rodents are more active - has previously been shown effective to enhance neuronally released cortical acetylcholine levels. Nevertheless, perfusion of the microdialysis probe, inserted in the hippocampus of rats habituated to a reversed day-night rhythm, with a Ringer's solution without Ca^{2+} ions or containing the voltage-dependent Na^+-channel blocker tetrodotoxin during the dark phase did not affect hippocampal dialysate glutamate levels. These pharmacological manipulations, commonly used to verify vesicular release, produced a pronounced attenuation of hippocampal dopamine dialysate levels in the same

experimental conditions. This demonstrates that maintenance of basal hippocampal glutamate levels is predominantly Ca^{2+}- and Na^{+}-independent. However, reverse microdialysis of non-selective inhibitors of system x_c^-, *(RS)*-2-chloro-5-hydroxyphenylglycine (CHPG) or LY367385, significantly decreased hippocampal glutamate levels. This CHPG- or LY367385-induced attenuation of extracellular glutamate is more likely to originate from inhibition of system x_c^- rather than their $mGluR_{1/5}$ antagonist properties, given that reverse microdialysis of the $mGluR_{1/5}$ antagonist AIDA failed to alter hippocampal glutamate concentrations.

Stimulation of glutamate/cystine exchange via system x_c^- enhances glutamate levels in hippocampus

Reverse dialysis of N-acetylcysteine (N-Ac), a precursor of cysteine known to activate system x_c^-, significantly enhanced the extracellular hippocampal glutamate levels as measured with microdialysis in conscious rats.

III.2. Progress made in work package 4, acute seizure models

No spontaneous epileptic seizures were detected in $xCT^{-/-}$ mice

Basal electroencephalographic (EEG) activity obtained from the cortex of $xCT^{+/+}$ and $xCT^{-/-}$ mice was monitored at least 5 hours a day during an entire month. Every 2 days, the basal EEG of the mice was measured overnight for at least 12 hours. These EEG recordings did not show any appearance of cortical abnormalities in the $xCT^{-/-}$ mice and were comparable to the baseline EEG spectra of $xCT^{+/+}$ littermates. Focal seizure activity within specific brain sites cannot be excluded by these recordings from the cortical surface.

$xCT^{-/-}$ mice are less susceptible to limbic seizures with secondary generalization

Infusion of pilocarpine or kainic acid into the lateral tail vein of mice produced an array of rapidly progressing behaviours. Both chemoconvulsants induced limbic seizures with secondary generalization in mice though with a different behavioural convulsion pattern.

Pilocarpine infusion (i.v.) induced head bobbing, bilateral forelimb clonus with rearing, followed by clonic convulsions with loss of righting reflexes (falling), tonic hindlimb extension and death in all mice. Yet, a significantly higher dose of pilocarpine was necessary to induce these behaviours in $xCT^{-/-}$ mice compared to $xCT^{+/+}$ littermates. We revealed a significant effect of genotype upon the threshold dose of pilocarpine for inducing bilateral myoclonus, clonic convulsions with loss of righting reflexes, tonic hindlimb extension and death.

Kainic acid infusion induced behavioural arrest, followed by falling, clonic convulsions, tonic hindlimb extension and death in all mice. The dose of kainic acid, necessary to induce the consecutive typical behaviours, was however significantly higher in the $xCT^{-/-}$ mice compared to their $xCT^{+/+}$ littermates. This effect reached significance for behavioural arrest, falling, clonic phase, tonic phase and death.

I.p. injection of a third chemoconvulsant, *NMDA*, at a dose of 100 and 125 mg/kg, resulted in a mortality rate of respectively 13% and 71% in $xCT^{+/+}$ mice whereas in $xCT^{-/-}$ mice the mortality was respectively 0% and 20%. Moreover, the initial convulsion time, which is the time until the first convulsion with a score 3, was significantly higher in $xCT^{-/-}$ mice compared to $xCT^{+/+}$ mice. Also the average convulsion score was significantly smaller in $xCT^{-/-}$ mice compared to their wildtype littermates.

Stimulation of glutamate/cystine exchange via system x_c^- is proconvulsive

C57Bl/6 mice treated with N-acetylcysteine (N-Ac, 90 mg/kg i.p.), a cyst(e)ine prodrug, 90 min before the start of pilocarpine infusion via the tail vein, were significantly more susceptible to pilocarpine-induced head bobbing, rearing, clonic convulsions with loss of righting reflexes, tonic hindlimb extension and

death. The proconvulsant effect of N-Ac was completely abolished in the $xCT^{-/-}$ animals. No difference was observed in susceptibility for pilocarpine thresholds between N-Ac-treated and saline-injected $xCT^{-/-}$ animals.
The proconvulsant effect of N-Ac was also demonstrated in the 6Hz electrical corneal stimulation seizure model. In mice treated with N-Ac (90 mg/kg i.p.), 6Hz stimulation currents of 11 mA induced seizures in 25% of the mice. Increasing current intensity stepwise to a level of 28 mA induced seizures in all N-Ac-treated mice. Higher stimulation intensities were required to induce seizures in the saline-treated mice. A stimulation intensity of 53 mA was necessary to induce seizures in all saline-treated animals. A stimulation current of 28 mA induced seizures in 2 out of 5 control mice, while 14 mA did not induce seizures in any of the saline-treated mice. The CC_{50} values are 15.81 mA for N-Ac-treated mice and 33.23 mA for saline-treated mice.

III.3. Progress made in work package 5, learning and memory paradigms

Normal open field behaviour of $xCT^{-/-}$ mice

There are no genotype differences between young or old $xCT^{-/-}$ and $xCT^{+/+}$ mice for the distance travelled, the average movement speed and the percentage of time spent in the centre of the open field. This indicates that $xCT^{-/-}$ mice do not have gross motor dysfunction, motivational deficits for exploring a novel environment or increased aversion for the centre of an open field. An overall effect of age was observed for the travel distance and movement speed but not for time spent in the centre of the open field.

Intact spatial reference memory in $xCT^{-/-}$ mice tested in a Morris water maze

Escape latency analysis for *visible platform* testing in a water maze in young and old mice revealed no significant main effect of genotype, no significant genotype-by-day interaction, but a highly significant effect of trial day in both young and old mice, indicating that mice with xCT deletion were equally efficient as their $xCT^{+/+}$ controls at learning the non-spatial aspects of the water maze task. Swimming speed genotype effects and genotype-by-day effects were not significant during visible platform testing in young and old mice.

In the *hidden platform* setup, escape latency analysis revealed no effect of genotype, no genotype-by-day interaction but a highly significant effect of trial, demonstrating that both young and old $xCT^{-/-}$ and $xCT^{+/+}$ mice were capable of learning the spatial memory task in the water maze. Nevertheless, young $xCT^{-/-}$ mice showed a tendency of learning the hidden platform water maze task less efficiently. Swimming speed genotype effects and genotype-by-day interactions were not significant during hidden platform testing in young or old mice.
When comparing the average escape latencies of the first and last trial within a day for the first three days of hidden platform testing we observed no genotype effect or genotype-by-trial interaction. The overall effect of trial number was significant in young mice and borderline significant in old mice. This indicates that task performance improves comparably within a day for both genotypes. When comparing the average escape latencies of the last trial of a day and the first trial of the following day for the first three days of hidden platform testing we observed no genotype effect, genotype-by-trial interaction or trial effect. This indicates that task performance does not deteriorate significantly between consecutive trial days and suggests intact reference memory in young and old mice of either genotype.
Retention of spatial search strategies was evaluated in young and old mice in a *probe trial* performed 2 hours following the final hidden platform training session. We revealed no genotype effects or genotype-by-target quadrant interactions but both young and old $xCT^{-/-}$ and $xCT^{+/+}$ mice showed a clear preference for the target quadrant. This further demonstrates that spatial reference memory is intact in young and old $xCT^{-/-}$ and $xCT^{+/+}$ mice.

Impaired spatial working memory in xCT$^{-/-}$ mice tested in a Y-maze

In the *delayed Y-maze spontaneous alternation* task, used as a measure for spatial short-term memory, we observed a significant target arm effect, with both young and old mice making more entries in the novel arm of the Y-maze following a previous exposure to two familiar arms. A significant genotype-by-target arm interaction was observed in young mice, with xCT$^{-/-}$ mice making less entries into the novel arm compared to xCT$^{+/+}$ mice, but not in old mice. Further analysis of the number of arm entries showed no main effect of genotype in either young mice or old mice. When analyzing dwell times in each of the Y-maze arms, no significant main effect of genotype or genotype-by-target arm interaction was observed in either young or old mice. Young xCT$^{-/-}$ and xCT$^{+/+}$ littermates spent significantly more time in the novel arm of the Y-maze. However, the dwell-time preference of old xCT$^{-/-}$ and xCT$^{+/+}$ mice for the novel arm of the Y-maze was not significant.

In the *continuous Y-maze spontaneous alternation* task, an overall genotype effect was observed for the alternation score with young xCT$^{-/-}$ mice making less correct alternations compared to young xCT$^{+/+}$ mice. We observed no significant age effect or genotype-by-age interaction. No genotype effect, genotype-by-age interaction or age effect was observed for the number of arm entries made by young or old xCT$^{+/+}$ and xCT$^{-/-}$ mice. This indicates that the decrease in alternation score in young xCT$^{-/-}$ mice was due to a spatial working memory deficit rather than a deficit in locomotor activity or exploratory motivation.

III.4. Summary

System x_c^- exchanges intracellular glutamate for extracellular cystine, giving it a potential role in intracellular glutathione synthesis and non-vesicular glutamate release. We report that mice lacking the specific xCT subunit of system x_c^- (xCT$^{-/-}$) do not have a lower hippocampal glutathione content, increased oxidative stress or brain atrophy, nor exacerbated spatial reference memory deficits with ageing. Together these results indicate that loss of system x_c^- does not induce oxidative stress *in vivo*. Young xCT$^{-/-}$ mice did however display a spatial working memory deficit. Interestingly, we observed significantly lower extracellular hippocampal glutamate concentrations in xCT$^{-/-}$ mice compared to wildtype littermates. Moreover, intrahippocampal perfusion with system x_c^- inhibitors lowered extracellular glutamate whereas the system x_c^- activator N-acetylcysteine elevated extracellular glutamate in the rat hippocampus. This indicates that system x_c^- may be an interesting target for pathologies associated with excessive extracellular glutamate release in the hippocampus. Correspondingly, xCT deletion in mice elevated the threshold for limbic seizures and abolished the proconvulsive effects of N-acetylcysteine. These novel findings sustain that system x_c^- is an important source of extracellular glutamate in the hippocampus. System x_c^- is required for optimal spatial working memory, but its inactivation is clearly beneficial to decrease susceptibility for limbic epileptic seizures.

IV. 2011 publication list

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

– D. De Bundel[#], A. Schallier[#], E. Loyens, R. Fernando, H. Miyashita, K. Vermoesen, J. Van Liefferinge, S. Bannai, H. Sato, **Y. Michotte**, **I. Smolders**[#], **A. Massie**[#]
(**[#]equally contributing authors**)
Loss of system x_c^- does not induce oxidative stress but decreases extracellular glutamate in hippocampus and influences spatial working memory and limbic seizure susceptibility.
J. Neurosci. 31 (2011) 5792-5803.
5-year SCI impactfactor = 8.068

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

E. Loyens, A. Schallier, S.Y. Chai, D. De Bundel, P. Vanderheyden, **Y. Michotte**, **I. Smolders**.
Deletion of insulin-regulated aminopeptidase in mice decreases susceptibility to pentylenetetrazol-induced generalized seizures.
Seizure. 20 (2011) 602-605.
5-year SCI impactfactor = 1.857

– E. Loyens, K. Vermoesen, A. Schallier, **Y. Michotte, I. Smolders**.
Proconvulsive effects of oxytocin in the generalized pentylenetetrazol mouse model are mediated by vasopressin 1a receptors.
Brain Res. 2011 Dec 7. [Epub ahead of print]
5-year SCI impactfactor = 2.665

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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 the interaction networks. In 2011 we made major breakthrough in distal hereditary motor neuropathies (distal HMN), hereditary sensory and autonomic neuropathies (HSAN) and in early onset hereditary motor and sensory neuropathies (HMSN) resulting in two PhD's and 12 publications acknowledging the GSKE.

In 2004, we were the first to report that distal HMN or the spinal form of CMT neuropathy can be caused by missense mutations in the small heat shock proteins HSPB1 and HSPB8 [Evgrafov *et al.* 2004;Irobi *et al.* 2004]. More recently we found that some of the HSPB1 mutations display an enhanced affinity to client proteins [Almeida-Souza *et al.* 2010]. The most striking differential interacting protein was tubulin. We demonstrated that this anomalous binding leads to the stabilization of the microtubule network in a microtubule-associated protein (MAP)-like manner [Almeida-Souza et al. 2011]. In addition, we could confirm the enhanced interaction of mutant HSPB1 with tubulin in a transgenic mouse model for mutant HSPB1 [d'Ydewalle *et al.* 2011]. The increased stability of the microtubule network was also clear in neurons isolated from these mice. Since neuronal cells are particularly vulnerable to disturbances in microtubule dynamics, we hypothesized that this mechanism might explain the neuron-specific CMT phenotype caused by HSPB1 mutations [Almeida-Souza et al. 2011]. The clinical implications of our findings were recently highlighted in a review by Holzbaur and Scherer in NEJM [Holzbaur and Scherer 2011]. This work finally resulted in a PhD thesis by Leonardo Almeida-Souza (PhD defense was in December 2011). Our research is currently focused towards the study of other differential interaction partners to the mutant small heat shock proteins.

The main pathological feature of HSAN is a progressive degeneration of predominantly sensory and autonomic neurons, causing ulcero-mutilations in combination with variable autonomic and motor disturbances. Molecular genetics allowed to map and identify disease-causing mutations in ten genes and studies on the implications on protein function are rapidly following. While some of these proteins, such as nerve growth factor (NGF) and its receptor, have obvious nervous system-specific tasks, others are ubiquitously expressed proteins with housekeeping functions in the sphingolipid metabolism, vesicular transport, regulation of transcription or structural integrity. Mutations in *SPTLC1* and *SPTLC2*, encoding the two subunits of serine palmitoyltransferase (SPT), the enzyme catalyzing the first and rate-limiting step in the *de novo* synthesis of sphingolipids, have been reported to cause autosomal dominant HSAN type I (HSN-1) [Bejaoui *et al.* 2001;Dawkins *et al.* 2001;Rotthier *et al.* 2010]. We have demonstrated that two novel *SPTLC1* mutations result in a reduction of the SPT activity *in vitro*, and are associated with increased levels of the deoxysphingoid bases 1-deoxy-sphinganine and 1-deoxymethyl-sphinganine in patients' plasma samples. Our results confirm that the increased formation of deoxysphingoid bases is a key feature for HSAN type I as it was associated with all pathogenic *SPTLC1* and *SPTLC2* mutations reported so far, but also warrant for caution in the interpretation of *in vitro* data [Rotthier *et al.* 2011].

So far mutations in the anonymous transcript *FAM134B* [Kurth *et al.* 2009] and the HSN2-exon of the *WNK1* gene [Lafrenière *et al.* 2004] were associated with HSAN type II, a rare autosomal-recessive HSAN subtype. In collaboration, we contributed to the identification of a third gene for HSAN type II. Through genome-wide homozygosity mapping we identified a region located on chromosome 2q37.3 spanning the *KIF1A* gene. Subsequent sequencing of KIF1A in a series of 112 unrelated patients (including our HSAN patient cohort reported before [Rotthier *et al.* 2009]) revealed truncating mutations in three HSAN families. The KIF1A protein encodes an axonal transporter of synaptic vesicles, and interacts with the domain encoded by the HSN2-exon of *WNK1*. Similarly to *WNK1* mutations, pathogenic mutations in *KIF1A* were almost exclusively restricted to an alternatively spliced exon [Riviere *et al.* 2011]. Our study provided additional insights into the molecular pathogenesis of recessive HSAN and highlighted the potential biological relevance of alternative splicing in the sensory nervous system.

Early onset HMSNs are rare disorders encompassing congenital hypomyelinating neuropathy (CHN) with disease onset in the direct post-natal period, and Dejerine–Sottas syndrome (DSS) starting in infancy. *De novo* mutations in the peripheral myelin protein *PMP22*, *MPZ* and *EGR2* genes are known to be a typical cause of early onset HMSN. In addition, mutations in other genes for CMT may lead to similar phenotypes. To estimate the mutation frequencies, and to gain insights into the genetic and phenotypic heterogeneity of early onset HMSN, we studied a large cohort of unrelated patients with a peripheral neuropathy starting within the first year of life. We performed a systematic mutation screening by direct Sanger sequencing of 11 genes (*MFN2, PMP22, MPZ, EGR2, GDAP1, NEFL, FGD4, MTMR2, PRX, SBF2* and *SH3TC2*). In addition, screening for the CMT1A duplication on chromosome 17p11.2-12 was performed. In 35 patients (representing 45% of the cohort), mutations were identified. Mutations in *MPZ, PMP22* and *EGR2* were found in patients with early hypotonia and breathing difficulties. The recessive genes (*FGD4, PRX, MTMR2, SBF2, SH3TC2* and *GDAP1*) were mutated in patients having early foot deformities and variable delay in motor milestones. Several patients with congenital foot deformities but an otherwise normal early development carried the CMT1A duplication. Our genotype/phenotype correlation illustrated the genetic heterogeneity underlying hereditary neuropathies with infantile onset [Baets *et al.* 2011].

Recently, dominant mutations in the non-selective calcium channel TRPV4 were shown to cause various dominantly inherited axonal neuropathies [Auer-Grumbach *et al.* 2010;Deng *et al.* 2010;Landoure *et al.* 2010]. TRP channels are cellular sensors fulfilling divergent roles in different organ systems in response to various chemical and physical stimuli. The normal function of TRPV4 in the peripheral nervous system remains poorly understood. The phenotypic variability of neuropathies caused by *TRPV4* mutations is extensive and clinically distinct axonal neuropathies, such as scapuloperoneal spinal muscular atrophy (SPSMA), CMT type 2C (CMT2C, with vocal cord paralysis), congenital distal spinal muscular atrophy (SMA) and undifferentiated forms of axonal CMT [Baets and Timmerman 2011]. We reported a family transmitting a *TRPV4* mutation (Arg269Cys); the proband presented with a SPSMA, whereas her symptomatic daughter suffered from severe congenital distal SMA. Electrophysiological evaluation revealed a pure axonal motor neuropathy. We also found that non-penetrance may be an integral feature of the neuropathic syndromes associated with *TRPV4* gene mutations [Berciano *et al.* 2011]. Most genotype/phenotype correlations performed in the frame of this project resulted in the PhD thesis of Jonathan Baets, MD (PhD defense was in June 2011). Other relevant publications in the frame of the GSKE project can be found in our publication list.

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2. Research Activities:

Articles in International Journals – Acknowledging the GSKE:

- Rivière,J-B., Ramalingam,S., Lavastre,V., Shekarabi,M., Holbert,S., Lafontaine,J., Srour,M., Merner,N., Rochefort,N., Hince,P., Gaudet,R., Mes-Masson,A-M., Baets,J., Houlden,H., Brais,B., Nicholson,G., Van Esch,H., Nafissi,S., **De Jonghe,P.**, Reilly,M., **Timmerman,V.**, Dion,P.A., Rouleau,G.A.: KIF1A, an axonal transporter of synaptic vesicles, is mutated in hereditary sensoryand autonomic neuropathy type 2. American Journal of Human Genetics 89(2): 219-230 (2011) (Impact Factor: 11.680)
- Baets,J., Deconinck,T, De Vriendt,E., Zimon,M., Yperzeele,K., Van Hoorenbeeck,L., Peeters,K., Spiegel,R., Parman,Y., Ceulemans,B., Van Bogaert,P., Pou-Serradell,A., Bernert,G., Dinopoulos,A., Auer-Grumbach,M., Sallinen,S.-L., Fabrizi,G.-M., Pauly,F., Van den Bergh,P., Bilir,B., Battaloglu,E., Madrid,R., Kabzinska,D., Kochañski,A., Topaloglu,H., Miller,G., Jordanova,A., **Timmerman,V.**, **De Jonghe,P**.: Genetic spectrum of hereditary neuropathieswith onset in the first year of life. Brain 134(Pt 9): 2664-2676 (2011) (Impact Factor: 9.232)
- Baets,J., **Timmerman,V.**: Scientific Commentary: Inherited peripheral neuropathies: a myriad of genes and complex phenotypes Brain 134: 1585-1590 (2011) (Impact Factor: 9.232)
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- Baets,J., **De Jonghe, P.:** Editorial: TRPV4 neuropathies: calcium channel inhibition as a therapeutic target? Neurology 76(10): 856-857 (2011) (Impact Factor: 8.017)

Scientific Prizes:

- **J. Baets**: Flemish Foundation for Neurology, Eli-Lilly research prize 2010, Leuven, Belgium, March 19, 2011
- **L. Almeida-Souza**: Belgian Society of Biochemistry and Molecular Biology, Young Scientist Poster Prize, Belgian Society of Biochemistry and Molecular Biology 204th Meeting – Redox Mechanisms, Poster 'Unravelling the redox regulation of the small heath shock protein HSP1', Brussels, Belgium, May 6, 2011

Awards and fellowships:

- **L. Almeida-Souza:** Peripheral Nerve Society, PNS Travel Award, Biennial Meeting of the Peripheral Nerve Society 2011, Potomac, Maryland, USA, June 25-29, 2011
- **K. Janssens:** Peripheral Nerve Society, PNS Travel Award, Biennial Meeting of the Peripheral Nerve Society 2011, Potomac, Maryland, USA, June 25-29, 2011

PhD theses:

- **J. Baets: "**Genotype-phenotype correlations in hereditary neuropathies: a systematic approach", Promotor: De Jonghe P., Timmerman V., June 6th 2011
- **L. Almeida-Souza: "**Ubiquitous presence, local damage: The role of HSPB1 in the biology and disease of the peripheral nervous system", Promotor: Timmerman V. & Janssens S., December 13th 2011

Master theses:

- **De Cleir J.:** "Effect van RAB7- en SPTLC2-mutaties op axonaal transport en axonale degeneratie", Supervisor: Janssens K. (Academic MSc Thesis Biochemistry & Biotechnology, UA)
- **Geuens T.:** "Characterization of a novel binding partner of mutant HSPB1, involved in the peripheral neuropathy of Charcot-Marie-Tooth", Supervisors: Janssens S. & Timmerman V. (Academic MSc Stage Biochemistry & Biotechnology, UA)
- **Peeraer L.:** "De rol van TLR1 bij acute neurodegenerative in het perifere zenuwstelsel", Supervisors: Janssens S. & Timmerman V. (Academic MSc Thesis Biomedical Sciences, UA)
- **Vermeylen S.:** "Optimalisatie en studie van cellulaire modelsystemen voor mutaties in small heat shock protein HSPB1 die leiden tot CMT", Supervisor: Timmerman V., (Academic MSc Stage Biochemistry & Biotechnology, UA)
- **Vermeylen S.:** "Search for protein interactors of SH3TC2, a protein mutant in Charcot-Marie-Tooth neuropathy", Supervisors: Timmerman V. & Palau F. (Academic MSc Thesis Biochemistry & Biotechnology, Erasmus Valencia)
- **Krols M.:** "The role of mammalian Target Of Rapamycin (mTOR) in Schwann cell development and peripheral myelination", Supervisors: Timmerman V. & Brophy P. (Academic MSc Thesis Biochemistry & Biotechnology, Erasmus Edinburgh)
- **Cottenie E.:** "Investigation of hereditary axonal neuropathies", Supervisors: De Jonghe P. & Reilly M. (Academic MSc Thesis Biochemistry & Biotechnology, Erasmus London)

Invited Lectures:

- **De Jonghe P.:** "CMT with early onset", 9th Congress of the European Paediatric Neurology Society; Cavtat, Croatia, May 11-14, 2011.
- **Timmerman V.:** "Molecular Genetics of Charcot-Marie-Tooth neuropathies", Italian Institute of Technology (IIT), seminar upon invitation by Dr. M. Pennuto, Genova, Italy, March 2, 2011
- **Timmerman V.:** "New molecular targets in hereditary neuropathies", UK Neuromuscular Translational Research Conference 2011, MRC Center for Neuromuscular Disease, London, UK, March 29-30, 2011
- **Timmerman V.:** Prof. Dr. P.K. Thomas Inaugural Lecture: "Molecular Genetics of Charcot-Marie-Tooth neuropathies: from mutations to gene interaction networks", 2011 PNS Biennial Meeting of the Peripheral Nerve Society, Potomac, Washington, USA, June 25-29, 2011
- **Timmerman V.:** "Update in CMT, distal SMA (HMN) and overlapping phenotypes, Recent Genetic Advances in Motor Neuron Diseases: Promises and Hurdles to Clinical Interventions", 12th International Congress of Human Genetics/ joint ASHG meeting, October 11-15, 2011
- **Timmerman V.:** "Molecular Genetics of Charcot-Marie-Tooth neuropathies: from mutations to gene interaction networks", 16th International Congress of the World Muscle Society, Almancil, Portugal, October 18-22, 2011
- **Timmerman V.:** "From small heat shock protein mutations to future therapeutic approaches in distal hereditary motor neuropathies", seminar upon invitation by Prof. Dr. N. Lévy and Dr. V. Delague, Marseille, France, November 30th, 2011
- **Timmerman V.:** "Molecular Genetics of Charcot-Marie-Tooth neuropathies: from mutations to gene interaction networks" upon invitation by Prof. Dr. P. Van Den Bergh, LOK seminar, UCL, Sint-Lambrechts-Woluwe, December 12th, 2011

Slide presentations selected at international meetings:

- **Almeida-Souza L.:** "Peripheral neuropathy mutants stabilize microtubules and reveal a novel role for HSPB1 in microtubule nucleation", 4th International CMT consortium meeting, Potomac, MD, USA, June 29 – July1, 2011
- **Baets J.:** "The contribution of TRPV4 mutations to the genetic spectrum of undifferentiated HMN and early onset HMSN", The 4th International CMT Consortium, Potomac, Maryland, US, June 29-July 1, 2011
- **Baets J.:** "Genetic spectrum of hereditary neuropathies with onset in the first year of life", 90th meeting of the Belgian-Dutch Neuromuscular Study Club, Utrecht, The Netherlands, March 16, 2011
- **Holmgren A.:** "Increased neurofilament phosphorylation by CMT causing mutant HSPB1 leads to aberrant interaction with the axonal transport system", 91st Meeting of the Belgian-Dutch Neuromuscular Study Club, Sint-Lambrechts-Woluwe, Belgium, September 28, 2011
- **Ipek F.:** "Modelling a sensory neuropathy caused by mutations in SPTLC2 in Drosophila", 91st Meeting of the Belgian-Dutch Neuromuscular Study Club, Sint-Lambrechts-Woluwe, Belgium, September 28, 2011
- **Irobi J.**: "Mutant HSPB8 causes motor neuron specific neurite degeneration", 4th international CMT consortium meeting, Potomac, MD, USA, June 29 – July 1, 2011
- **Ydens E.:** "Acute neurodegeneration triggers an alternative macrophage response", 91st Meeting of the Belgian-Dutch Neuromuscular Study Club, Sint-Lambrechts-Woluwe, Belgium, September 28, 2011

Poster presentations at international meetings:

- **Holmgren A.: "**Increased neurofilament phosphorylation by CMT causing mutant HSPB1 leads to aberrant interaction with the axonal transport system", 4th International CMT Consortium, Potomac, Maryland, United States of America, June 29-July 1, 2011
- **Janssens K.:** "HSAN type I caused by mutations in SPTLC1 and SPTLC2 is consistently associated with reduced SPT activity in vitro and formation of deoxysphingoid bases in vivo", Peripheral Nerve Society meeting, Potomac, MD USA, June 25-29 2011
- **Ydens E.:** "Acute neurodegeneration triggers an alternative macrophage response, 10th European meeting on Glial Cells in Health and Disease", Czech Republic, Prague, September 13-17, 2011

Slide presentations selected at national meetings:

- **Holmgren A.:** "Increased neurofilament phosphorylation by CMT causing mutant HSPB1 leads to aberrant interaction with the axonal transport system", IAP P6/43 Meeting, Sint-Lambrechts-Woluwe, October 24, 2011
- **Irobi J.:** "Mutant HSPB8 causes motor neuron specific neurite degeneration", 9th bi-annual Meeting of the Belgian Society for Neuroscience, Leuven, May 23, 2011
- **Janssens K.:** "Mutations in SPTLC1 and SPTLC2 cause HSAN type 1 by reducing SPT activity and producing atypical deoxyspinghoi bases", IAP P6/43 Meeting, Sint-Lambrechts-Woluwe, October 24, 2011

Poster presentations at national meetings:

- **Almeida-Souza L.:** "Unravelling the redox regulation fo the small heath shock protein HSP1. Belgian Society of Biochemistry and Molecular Biology 204th Meeting – Redox Mechanisms, Brussels, May 6, 2011
- **Ipek F.:** "Modeling a sensory neuropathy caused by mutations in SPTLC2 in *Drosophila*", IAP P6/43 Meeting, Sint-Lambrechts-Woluwe, October 24, 2011
- **Ydens E.:** "Acute neurodegeneration triggers an alternative macrophage response", IAP P6/43 Meeting, Sint-Lambrechts-Woluwe, October 24, 2011

Societal activities on national meetings:

- **Bouhy D.:** "Modéliser les neuropathies périphériques pour mieux les comprendre et les soigner". Merci Téléthon 2011, Association Belge contre les Maladies neuro-Musculaires, Forchie, November 29, 2011
- **Timmerman V.:** "Staan onderzoek en ontwikkelen van medicatie los van elkaar?", Nationale Studie- en Contactdag van CMT België v.z.w, Antwerpen, March 2, 2011

Progress report of the research group of

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Celsr genes in brain development and function

State of the art

Celsr (Cadherin, EGF-like, Laminin G-like, Seven pass, G-type Receptor) are developmentally regulated proteins with the potential to signal by homophilic interaction and/or heterophilic "ligand/receptor" interactions (Shima et al., 2007). Functional studies in the fruit fly have demonstrated a role for Flamingo, the *Drosophila* Celsr, in the orientation of epidermal structures, such as wing hairs (Chae et al., 1999; Usui et al., 1999), abdominal bristles (Lawrence et al., 2004) and the facets of the eye (Das et al., 2002). These structures are oriented within the plane of the epithelium, orthogonal to the apico-basal polarity axis, by a process referred to as planar cell polarity (PCP). Establishment of PCP during hair development in *Drosophila* is characterized by enrichment of Flamingo protein at proximal/distal cell boundaries (Lawrence et al., 2004; Usui et al., 1999). Celsr/Flamingo conveys polarity information by recruiting Frizzled and Dishevelled to one side, Van Gogh and prickle to the opposite side of the cell (Chen et al., 2008; Strutt and Strutt, 2008)

When we started studying the mammalian Celsr genes, two members (*Celsr1* and *Celsr2*) were identified, but nothing was known about their functions. We identified the third member (*Celsr3*) and studied expression of *Celsr1-3* during brain development. *Celsr1* is expressed in zones of neural progenitor proliferation (ventricular and subventricular zones). In the adult brain, expression is found in telencephalic subependymal zones (SEZ) and the subgranular layer in the dentate gyrus (SGL), two main niches of adult neural stem cells (NSC), as well as in cerebellar granule cells. This pattern suggests a function in neural stem cells and/or neurogenesis. *Celsr2* expression is diffuse in neural progenitors and strong in postmitotic neural cells. It is expressed at all stages, and remains high in the adult, suggesting a role in brain maintenance or plasticity. *Celsr3* is expressed in postmitotic neural cells and progressively downregulated during maturation, suggesting that it acts during neuronal differentiation and maturation. In the adult brain, Celsr3 is weakly expressed in NSC (Tissir et al., 2002; Tissir and Goffinet, 2006). Given the high and dynamic expression of *Celsr1-3* in the nervous system, we decided to investigate their roles in mice. Using a conditional *Celsr3* knock-out (KO), we showed that Celsr3 plays a crucial role in axon guidance and brain wiring (Tissir et al., 2005). Remarkably, the phenotype of *Celsr3*$^{KO/KO}$ mice is almost identical to that of *Frizzled3*$^{KO/KO}$, (one of the ten mammalian frizzled orthologs, (Wang et al., 2002). The fly Celsr/Flamingo and Frizzled belong to the core PCP group. Thus, this observation indicates that *Celsr3* and *Fzd3* are partners in a PCP-like mechanism, conserved from flies to mammals, that governs the development of axonal tracts (Tissir et al., 2005). This view is now widely accepted and has been extended to other developmental processes (Tissir and Goffinet, 2010). We next generated conditional and constitutive *Celsr1* mutants. We and others showed a role of Celsr1 in neural tube closure (Curtin et al., 2003; Ravni et al., 2009). Recently, we have shown that Celsr1 provides a directional cue for migration of facial branchiomotor neurons (FBMN) in the hindbrain (Qu et al., 2010). We obtained a *Celsr2* genetrap mutant and showed that Celsr2, together with Celsr3, is required for ependymal ciliogenesis and cerebrospinal fluid dynamics (Tissir et al., 2010). Celsr2 is also involved in migration and survival of facial branchiomotor neurons (Qu et al., 2010).

Role of Celsr1-3 in ependymal ciliogenesis

The wall of the lateral ventricles of the postnatal brain, hereafter referred to as lateral wall "LW", is a region where neural stem cells persist throughout life. The observation of the LW using « en face » whole mount preparations highlighted a strictly organized architecture (Mirzadeh et al., 2008). A striking feature of multiciliated ependymal cells is the high degree of their planar polarization (Mirzadeh et al., 2010). At the cell level, all cilia need to beat in the same direction. Therefore, their basal feet (lateral extensions of BB that point to the direction of the effective stroke of cilia beat) rotate during development and adopt a homogeneous orientation (rotational polarity). Planar polarity is also observed at the tissue scale: all ependymal cells display a shift of their basal bodies (BB) to the anterior side of the cell (translational polarity) (Guirao et al., 2010; Mirzadeh et al., 2010). This specific organization of the lateral wall is essential for cerebrobrospinal fluid (CSF) circulation and its modification might impair stem cell maintenance and effective neurogenesis (Sawamoto et al., 2006, Paez-Gonzalez et al., 2011).

We have shown that loss of function of Celsr2 and Celsr3 impairs ciliogenesis and leads to defective flow of CSF and lethal hydrocephalus. Mutant ependymal cilia never develop in normal numbers and display abnormalities in morphology, position, and planar organization. Ciliary basal feet are misoriented, 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. The conventional method to analyze rotational polarity is to investigate the orientation of the basal foot by transmission electron microscopy on tangential sections of ependymal cells. This method is tedious and time consuming. To speed up the study of LW in our mutants, we developed a new approach in which we combine gamma tubulin and Ac3 immunostainings. These two proteins localize at distinct positions in the BB and define a vector that can be revealed by confocal microscopy and used to evaluate the rotational polarity. Using this method, we confirmed that rotational polarity of ependymal cells is perturbed in Celsr2 mutant mice. Celsr3 and Vangl2 null animals die shortly after birth precluding analysis in mature mice. To study the contribution of these genes to LW polarization, we are using a conditional deletion approach. We have Celsr3 and Vangl2 floxed alleles (*Celsr3*$^{f/f}$ and *Vangl2*$^{f/f}$) and have imported the *Nestin-CreERT2* line, in which the Cre recombinase is expressed under the control of Nestin promoter upon tamoxifen injection. We generated *Celsr3*$^{+/-}$; *Nestin-CreERT2* and *Vangl2*$^{+/-}$; *Nestin-CreERT2* males that we are crossing with Celsr3$^{f/f}$ and Vangl2$^{f/f}$ females respectively. Tamoxifen is injected intraperitonealy to E13.5 pregnant dames and the progeny is analyzed at P21.

Celsr1 and *Celsr2* mutants are viable. To investigate the potential role of Celsr1 and Celsr2 in translational polarity, we performed immunostaining on LW whole-mounts. We used antibodies against ZO1 and gamma tubulin which label tight junctions and basal bodies respectively. We analyzed the position of BB patch relative to the center of the cell. In all genotypes analyzed thus far, ependymal cells showed a displacement of BB. However, while in WT animals, all the BB patch are systematically shifted toward the anterior pole, Celsr1 mutant mice display abnormal translational polarity with BB clusters dispersed in any pole of ependymal cells. *Celsr2*$^{KO/KO}$ mice also have a slight defect of their translational polarity. It has been suggested that the primary cilium of radial glial (RG) cells, the precursor of ependymal cells control the translational polarity in the later. We analyzed the presence of primary cilium in our mutants. Immunostaining against either gamma tubuline or acetylated tubulin demonstrated that virtually all RG cells bear a primary cilium at birth suggesting that the translational polarity defects observed in *Celsr1* and to a lesser extent in *Celsr2* mutants are not due to lack of the primary cilium. We then carried-out a time course analysis and found that the primary cilium is progressively polarized to the anterior side of

the cell in normal animals, anticipating ependymal cell translational polarity. In *Celsr1* mutant mice, BB of the primary monocilium migrates away of the center of the RG cells but not systematically toward the anterior side. The same phenotype is observed in mice mutant for *Fzd3*, another PCP gene. Interestingly RG polarity was not affected *Celsr3* mutants. All phenotypes described above have been quantified using a program that we developed to measure BB dispersal. Our results suggest that Celsr1 control the localization of primary cilium on the apical surface of RG cells. This localization could then affect the distribution of multicilia. Alternatively, the two functions of Celsr1 in translational polarity of RG on the one hand and ependymal cells on the other hand are independent. To test this hypothesis, we will use the *Celsr1^{f}* (foxed allele) to inactivate the gene when RG polarity is already established. Tamoxifen will be injected to pregnant dames at E18.5 and translational polarity of multicilia will be assessed at P21. *Celr1$^{+/-}$; NestinCreERT2* males have been generated and are being crossed with *Celsr1$^{f/f}$* females.

Cell polarization events often rely on cytoskeleton rearrangements. Looking for proteins that could link PCP to cytoskeleton, we decided to focus on the formins. These proteins form a family of 15 members known to impact on cytoskeleton components (Chesarone et al., 2010). Among them, Daam1 and Daam2 physically interact with Dishevelled, a core PCP protein (Habas et al., 2001). We studied the expression of formins by in situ hybridization at different embryonic and postnatal stages. Based on their high mRNA expression level in the LW, we selected 3 genes namely, Daam2, Fmnl3 and Diap3. To knock out these genes in mouse, we electroporated ES cells and selected clones that had undergone homologous recombination and integrated the modified alleles. Recombinant ES cells were injected in blastocysts and chimeric mice “competent” for germline transmission were obtained for the three genes. The first generation of homozygous mice will be soon available. After validation by RT-PCR and western blotting, the knockout mice we will analyzed for polarity of LW.

In summary, during this year, we developed genetic and technological tools to study polarization of LW. We demonstrate that PCP genes control different aspects of LW polarity. The first set of results should be submitted for publication within 6 months.

Celsrs in neuronal migration

We recently reported that *Celsr1*, *Celsr2,* and *Celsr3* play essential roles in FBMN migration. Whereas *Celsr2&3* regulate the extent and trajectory of FBMN migration, *Celsr1* specifies the direction of migration in a non cell autonomous manner (Qu et al., 2010). To our knowledge, this is the first evidence that a protein regulates direction of FBMN migration along the rostro-caudal axis, without affecting the ability of neurons to migrate. The mechanism of action of Celsr1 is particularly intriguing, because inactivation in neural stem cells (NSC) impacts on the migration of daughter cells. Another example of neuronal migration along the rostro-caudal axis concerns NSC that are continuously generated in the subependymal zone and migrate to the olfactory bulb (OB) via the rostral migratory stream (RMS), thereby contributing to the ongoing renewal of interneurons in the OB. We found that *Celsr1* mutant mice display a marked atrophy of the OB (Fig. 1).

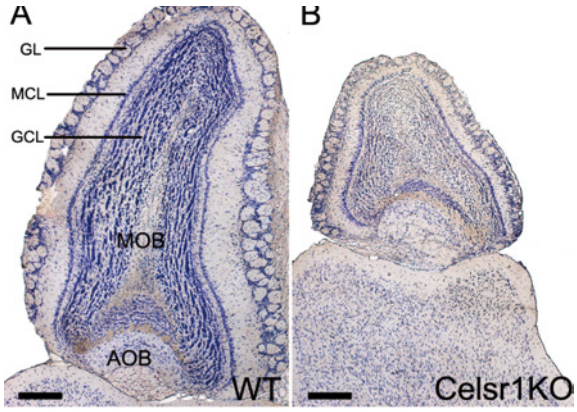

Figure 1: Nissl staining of wild-type (A) and *Celsr1* mutant (B) OB. The size of *Celsr1* mutant main olfactory bulb (MOB) is smaller than that in WT, while the accessory olfactory bulb (AOB) seems unaffected. The average diameter of individual glomeruli in mutant is significantly decreased when compared to its WT littermate. GL: glomerular cell layer, GCL: granule cell layer, MCL: mitral cell layer. Scale bar: 500 µm

To assess whether Celsr1 deficiency affects adult nurogenesis and/or migration of neuroblasts to the OB, we carried out immunostaining with nestin antibodies that label NSC. The number of nestin positive cells was similar in control and Celsr1 mutant mice, suggesting that neurogenesis is unaffected in the latter. We also showed that the number of BrdU positive cells after short post-BrdU survival time (1h), and the number of Ki-67 positive cells which reflects the number of cycling cells, are similar in both genotypes. We still need to compare the ratio of Ki-67 positive cells to BrdU positive cells after short post-BrdU survival time, to evaluate the length of cell cycle in Celsr1 deficient versus wild type mice.

Since Celsr1 loss of function does not seem to affect neurogenesis, we wondered whether it might interfere with migration of neuroblasts. Using an antibody against Doublecortin (Dcx) which specifically labels neuroblasts, we didn't see any difference in the total number of Dcx positive cells between the two genotypes. However, we found that these cells accumulate along the SVZ in *Celsr1*$^{KO/KO}$ mice, whereas most of them were found in OB and along the RMS in the WT. Hence, the number of Dcx positive cells in the center of OB was smaller in *Celsr1*$^{KO/KO}$ than that in WT (Fig 2).

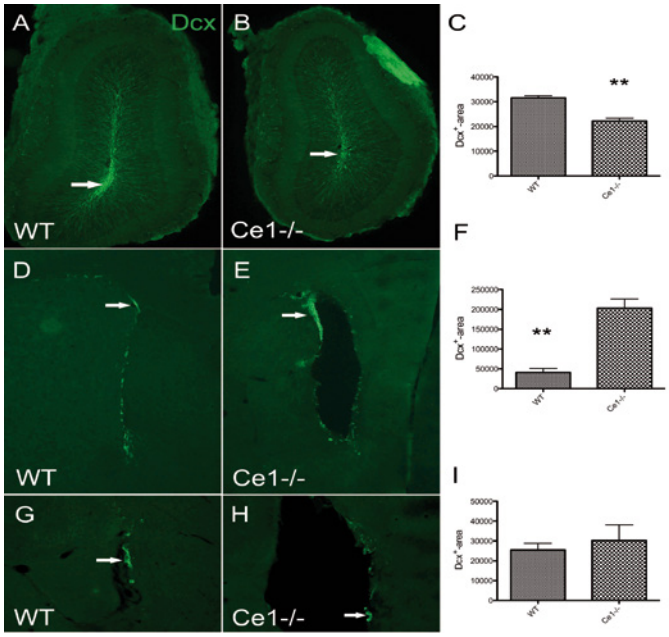

Figure 2. Distribution of Dcx positive cells. A, B: Dcx positive-cells in WT (A) and *Celsr1* mutant (B) OB; D, E: Dcx positive-cells in the rostral SVZ of WT (D) and *Celsr*1 mutant (E); G, H: Dcx positive-cells in the caudal SVZ of WT(G) and *Celsr1* mutant (H); C, F, I: Quantification of Dcx positive-cells in the OB (C), rostral SVZ (F) and caudal SVZ (I).

To estimate the number of migrating neuroblasts, we injected BrdU intraperitoneally for 3 consecutive days to WT and Celsr1 mutant mice and sacrificed them 1 week after the last injection. Immunostaining with a BrdU antibodies indicated that BrdU positive cells accumulated along the rostral tier of the lateral ventricle in Celsr1 mutants, which is similar to the result obtained with Dcx staining. Accordingly, the number of BrdU positive cells in the center of the OB was decreased in *Celsr1*$^{KO/KO}$ versus WT mice (Fig 3). Taken together, these results show that the rostral migration of neuroblasts is disturbed by *Celsr1* inactivation. Instead of moving rostrally to the OB, neuroblasts accumulate in the rostral part of the lateral ventricular wall in Celsr1 mutant. As a result, fewer cells reach their final destination in the OB, resulting into OB atrophy.

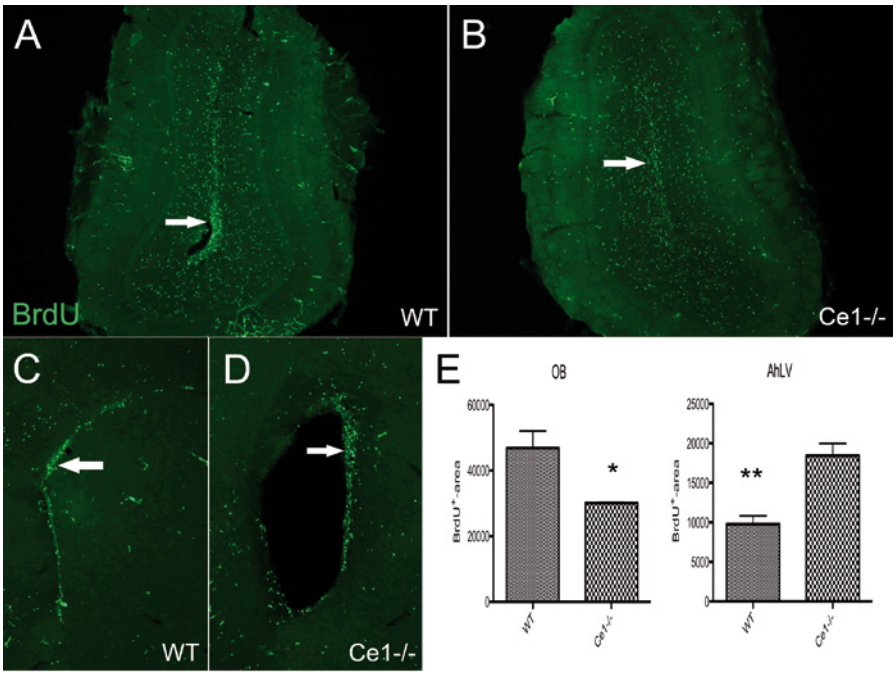

Figure 3. Distribution of BrdU positive cells. A, B: BrdU positive cells (arrows) in WT (A) and *Celsr1* mutant (B) OB. C, D: BrdU positive cells in the rostral part of WT (C, quantified in E left panel) and *Celsr1* mutant (D, quantified in E right panel) lateral ventricle.

How does Celsr1 mutation affect neuroblasts migration? To address this question, we undertook co-immunostaining experiments to know which cell types express Celsr1. We found that Celsr1partially co localizes with nestin, a marker of stem cell (B cells, Fig 4A) and Mash1, a marker of transit amplifying cells (C cells, Fig 5B). Furthermore, while no co-localization between Celsr1 and Dcx, a marker of neuroblasts was detected (Fig C), Celsr1 was heavily expressed with GFAP in astrocytes that form glial tunnels encompassing migrating neuroblasts (Fig 4D). These results suggest that, like in the embryonic hindbrain, Celsr1 regulates the migration of neurobalts in a non cell autonomous manner, most likely by affecting the astrocytic tunnels which channel the chains of migrating neuroblasts. To further test this hypothesis, we will pursue our investigations along different lines: i) we will examine the structure and the development of astrocytic tunnels in Celsr1 mutants; ii) we will culture explants of RMS on primary cultures of astrocytes isolated from WT and *Celsr1* mutant brains and evaluate the migration index in each genotype; iii) we will graft WT neuroblasts (by stereotaxic of injection of fluorescent neuroblasts in the subependymal zone) in WT and *Celsr1* mutant brains, scarify the animals after a period of one week and quantify the number of grafted neuroblasts that reach the OB; and iiii) We will inactivate *Celsr1* specifically in neuroblasts by crossing *Celsr1*^{f} with the *Dcx-Cre* mice that we have already imported. All these experiments should allow us to understand the function of Celsr1 in neuronal migration of neuroblats along the rostral-caudal axis.

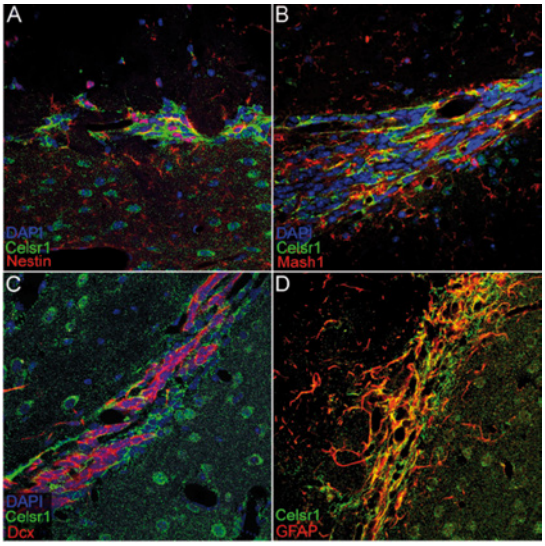

Figure 4. Expression of Celsr1 in the periventricular zone and the RMS. Brain sagitttal section were co-stained for Celsr1 and Nestin (A); Celsr1 and Mash1 (B); Celsr1 and Dcx (C); and Celsr1 and GFAP (D).

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

Introduction

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. Antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs), are important for the pathogenesis of MS, since they initiate immune responses upon interaction with and antigen presentation to naïve T cells. APCs are involved in multiple stages during MS pathology, making them important cells to study and possibly manipulate therapeutically. 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 involving its main animal model, experimental autoimmune encephalomyelitis (EAE).

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. Although little is still known about the involvement of NF-κB in CNS inflammation, 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).

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 (Boone *et al*., 2004; Hitotsumatsu *et al.,* 2008). Interestingly, *A20/TNFAIP3* has been identified in humans as a susceptibility locus for multiple immunopathologies including Crohn's disease, systemic lupus erythematosus and rheumatoid arthristis (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 identified *A20/TNFAIP3* as a susceptibility gene for multiple sclerosis (De Jager *et al*., 2009).

The aim of our GSKE-project is to understand the function, activation and regulation of NF-κB activation by 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. With these *in vivo* studies, we hope to contribute to the better understanding of the mechanisms that are implicated in the pathogenesis of MS and other neuroinflammatory conditions, and which may have implications for the treatment of these pathologies.

Results 1st project year

1. A20 conditional gene inactivation.

As A20-deficient mice die early after birth, we generated a conditional A20 allele, in order to study the role of A20 in adult mice. Conditional gene inactivation was achieved through use of the Cre/*LoxP* recombination system in which the targeted gene, *A20/Tnfaip3*, is flanked by *LoxP* consensus sites. Cell-specific deletion of A20 can now be achieved by crossing this conditional knockout mouse with transgenic mice expressing Cre recombinase in a tissue specific pattern (Fig. 1). Many of such Cre transgenic lines are well-characterized and widely available. For our studies, we use transgenic mice expressing Cre in different cell types of the CNS and the immune system, in order to inactivate A20 in these cells. The following transgenic strains have been used: Nestin-Cre transgenic mice expressing Cre in neuronal and glial progenitor cells, resulting in the complete deletion of *A20* in all cells of neuroectodermal origin (Fig. 1); Thy1-Cre transgenic mice expressing Cre in mature neurons; MOGi-Cre transgenic mice expressing Cre in mature myelinating oligodendrocytes; GFAP-Cre transgenic mice expressing Cre in astrocytes; CD4-Cre transgenic mice expressing Cre in T cells; LysM-Cre transgenic mice expressing Cre in myeloid cells (including microglia) and CD11c-Cre transgenic mice expressing Cre in dendritic cells (DCs).

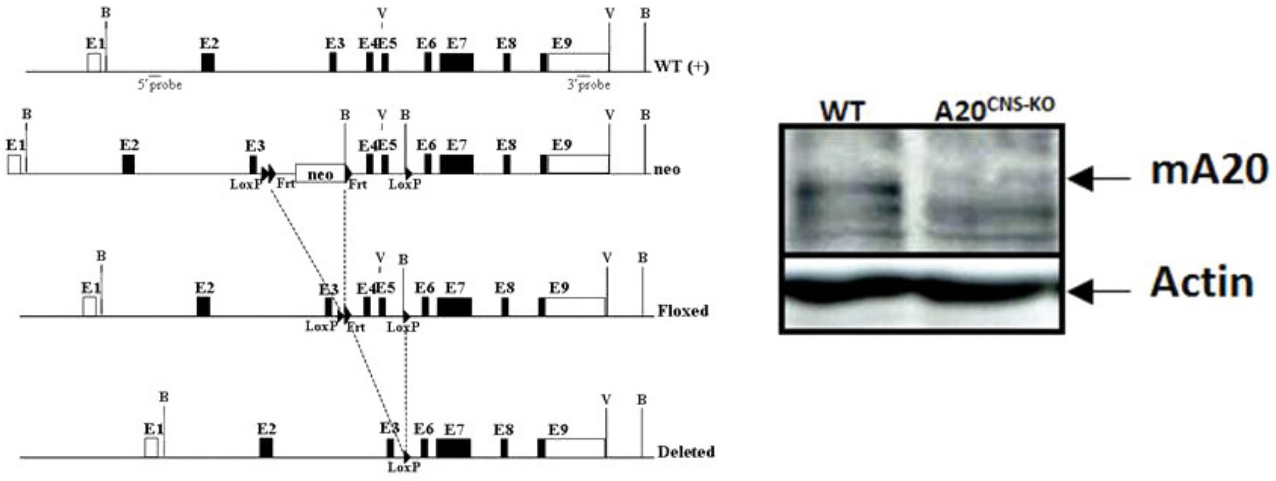

Figure 1. CNS-specific A20 deletion. A20 Targeting scheme. Diagram showing the wild-type (WT), targeted (neo), LoxP-flanked (Floxed) and the deleted A20 alleles. Boxes indicate exons 1 to 9 (E1-E9) (left). Western blot analysis of A20 and actin expression in wild-type (WT) and $A20^{CNS\text{-}KO}$ total brain lysate (right).

2. Mouse CNS disease models.

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, these different tissue-specific A20 knockout mice, together with control littermate mice, are 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 EAE, brain-specific demyelination can also be induced by putting mice on a diet containing the neurotoxicant cuprizone. Advantage of this approach is that demyelination can afterwards be reversed by administration of normal food, allowing the study of brain remyelination and the involvement of NF-κB in this.

3. Experimental Autoimmune Encephalomyelitis (EAE)

CNS-specific A20 knockout mice (A20$^{CNS\text{-}KO}$)

Expression of the Cre recombinase under control of the Nestin promoter allows the specific deletion of the floxed gene (A20) in all cells of neuroectodermal origin (all CNS excluding microglia). In contrast to most tissues where A20 is only expressed at low levels and is generally upregulated under inflammatory conditions, A20 is constitutively expressed in the CNS.

After immunization with MOG peptide, CNS-specific A20 knockout mice (A20$^{CNS\text{-}KO}$) show a similar disease onset, incidence and progression when compared to wild-type littermate mice (Fig. 2). These results demonstrate that the lack of A20 in all cells of neuroectodermal origin does not alter EAE. However, it might be that the effect of the lack of A20 in one cell type might counter the effect of lacking A20 in a different cell type, thus resulting in a net null effect.

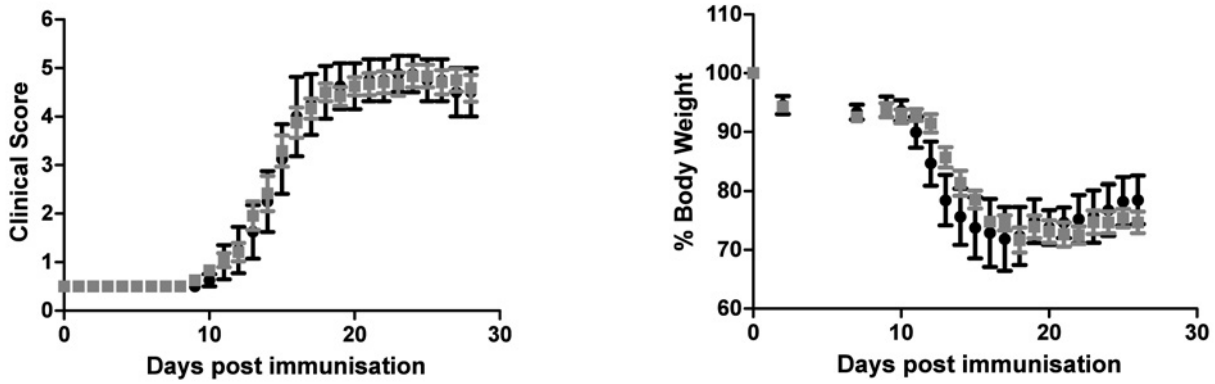

Figure 2. Clinical course of EAE in A20$^{CNS\text{-}KO}$ mice. Clincal disease score (left) and body weight (right) of A20$^{CNS\text{-}KO}$ mice (grey) and wild-type littermate mice (black) during the course of EAE.

Neuron-specific A20 knockout mice (A20$^{neur\text{-}KO}$)

Expression of the Cre recombinase under control of the neuronal Thy1.2 promoter allows the specific deletion of the floxed gene (A20) in neurons. When compared to control littermate mice, mice lacking A20 specifically in neurons (A20$^{neur\text{-}KO}$) develop a similar EAE disease pattern, reflected in similar onset, incidence and disease progression (Fig. 3).

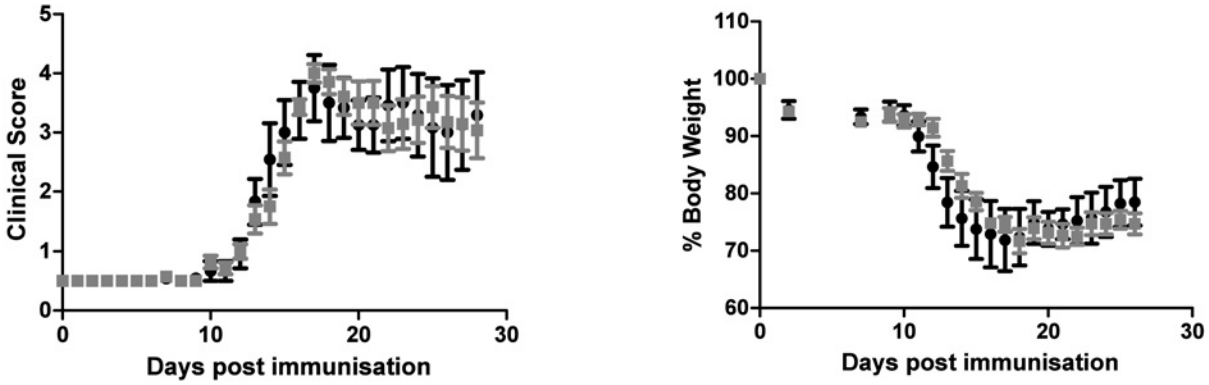

Figure 3. Clinical course of EAE in A20$^{neur\text{-}KO}$ mice. Clinical disease score (left) and body weight (right) of A20$^{neur\text{-}KO}$ mice (grey) and control littermate mice (black) during the course of EAE.

Oligodendrocyte-specific A20 knockout mice (A20$^{ODC\text{-}KO}$)

Expression of the Cre recombinase under control of the oligodendrocyte-specfic MOG promoter allows the specific deletion of the floxed gene in oligodendrocytes. Mice lacking A20 specifically in oligodendrocytes show an ameliorated clinical phenotype when compared to control littermates (Fig. 4). In particular, the disease incidence was reduced with approximately 30% in knockout mice. However, when neglecting these 'protected' mice, remaining knockouts show a similar disease progression compared to wild-type controls. These results suggest that oligodendrocyte specific A20 plays a role during the onset of EAE, but does not alter disease progression.

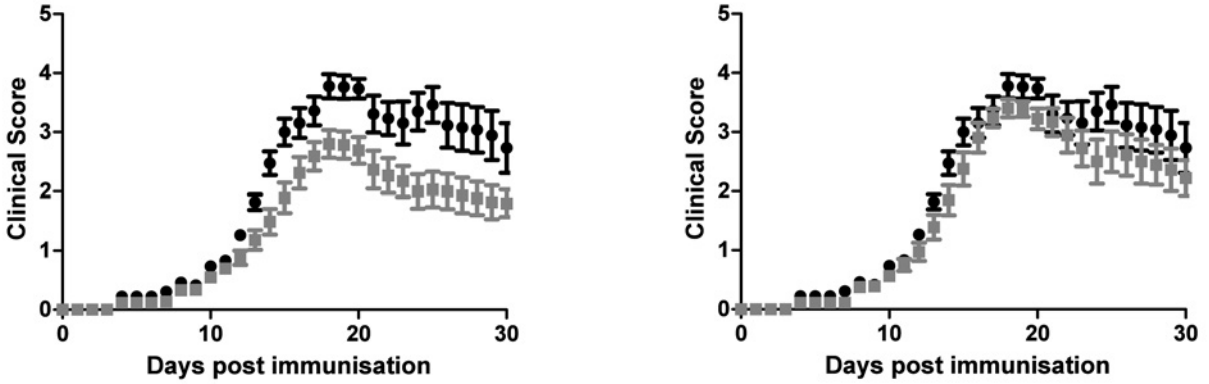

Figure 4. Clinical disease course of EAE in A20$^{ODC\text{-}KO}$ mice. Clinical disease score of A20$^{ODC\text{-}KO}$ mice (grey) and control littermates (black) during EAE, on a whole population (left) and when neglecting mice that don't get sick (right).

T cell-specific A20 knockout mice (A20$^{T\text{-}KO}$)

Expression of the Cre recombinase under control of the CD4 promoter allows the specific deletion of the floxed gene in T cells. In EAE, mice lacking A20 specifically in T-cells show a more severe clinical phenotype when compared to their wild-type littermate controls (Fig. 5). In an *in vitro* setting, purified A20 deficient CD4^{+} T-cells appear to show an enhanced responsiveness to stimulation with CD3/CD28, which is in correlation with the more aggressive disease pattern in these mice.

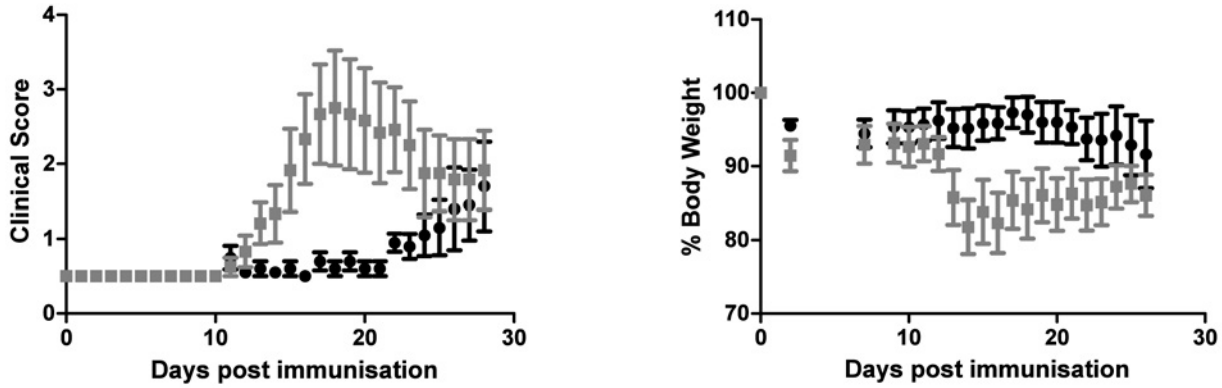

Figure 5. Clinical disease course of EAE in A20$^{T\text{-}KO}$ mice. Clinical disease score (left) and body weight (right) of A20$^{T\text{-}KO}$ mice (grey) and control littermates (black) during the course of EAE.

Myeloid cell-specific A20 knockout mice (A20$^{myel\text{-}KO}$)

Expression of the Cre recombinase under control of the LysM promoter allows the specific deletion of the floxed gene (A20) in all cells of myeloid origin, including macrophages and microglial cells. Mice lacking A20 in myeloid cells are completely protected against EAE (Fig. 6). These mice have an increased population of so-called 'myeloid-derived suppressor cells' (MDSCs), a heterogeneous population of immature CD11b+Gr1+ myeloid cells with immune suppressive activities. MDSCs have recently been shown to protect in EAE due to their capacity to mediate apoptosis of encephalogenic T-cells.

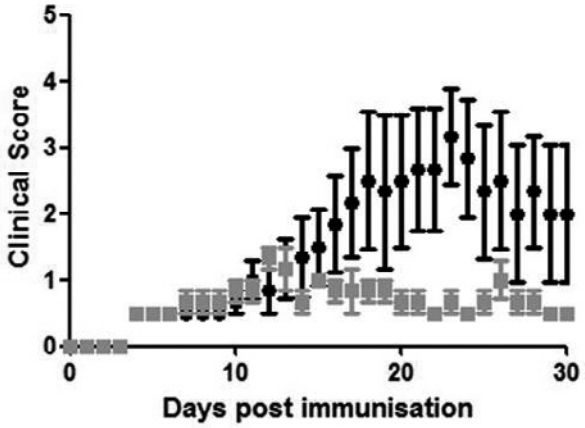

Figure 6. Clinical disease course of EAE in A20$^{myel\text{-}KO}$ mice. Clinical disease score of A20$^{myel\text{-}KO}$ mice (grey) and control littermates (black) during the course of EAE.

DC-specific A20 knockout mice (A20$^{DC\text{-}KO}$)

Expression of the Cre recombinase under control of the CD11c promoter allows the specific deletion of the floxed gene (A20) in dendritic cells (DCs). Mice lacking A20 in DCs are strongly protected against EAE, and only develop minor clinical pathology at later time points (Fig. 7). Similar to myeloid A20 knockout mice, these DC-specific A20 knockouts have expanded populations of MDSCs probably explaining their protective phenotype.

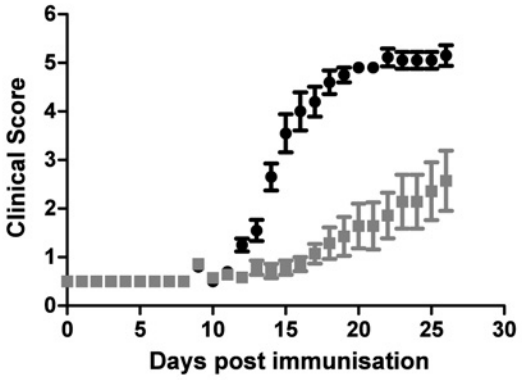

Figure 7. Clinical disease course of EAE in A20$^{DC\text{-}KO}$ mice. Clinical disease score of A20$^{DC\text{-}KO}$ mice (grey) and control littermates (black) during the course of EAE.

Future studies

During the second year of the project period, we will further study the molecular mechanisms explaining the phenotypes of the different cell-specific A20 knockouts in EAE. The capacity of macrophages and DCs in T cell activation will be analysed *in vitro* using primary cultures isolated from respective A20 knockout mice and control littermates. Similarly, primary neuronal cultures will be used *in vitro* to establish the impact of A20 deficiency on inflammatory challenge.

Next to the study of the role of A20 in EAE pathology, which is an autoimmune model for MS, demyelination can also be induced by treating mice with 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. In future studies, we will also focus on the role of NF-κB and A20 in the process of brain remyelination using this model. For this, the above described all CNS- and ODC-specific A20 knockout mice will be challenged with cuprizone to allow corpus callosum demyelination, after which remyelination will be studied changing mice to a regular diet.

Finally, our different brain-specific A20 knockout mice will also be subjected to cerebral ischemia, a model in which inflammation is strongly involved. There is strong evidence that NF-κB is activated in cerebral ischemia, mainly in neurons. Despite its well-known role as an antiapoptotic factor, in cerebral ischemia NF-κB contributes to neuronal cell death, at least if the ischemia is severe enough to lead to irreversible brain damage. In contrast, NF-κB also seems to be responsible for the preconditioning effect of a transient and sublethal ischemia, perhaps by dampening its own subsequent full activation (Ridder and Schwaninger, 2009). Since A20 mediates anti-inflammatory effects, by controlling NF-κB signalling, but also acts as a strong inhibitor of apoptosis, it may play important roles in the inflammatory and degenerative processes happening after brain ischemia.

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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.

One major problem in research relevant to brain diseases is the paucity of easily accessible neuronal cells of well defined subtypes, whether of animal or human origin. Embryonic stem cells, as well as recently developed induced pluripotent stem (iPS) cells, may offer new opportunities in this regard, given their unique properties of self renewal, maintained pluripotency, and potential availability in the human species. However it remains a major challenge to find efficient ways to generate defined cell types from these cells, which is a prerequisite for further use and validation of these stem cell-based systems in translational research.

Using basic know-how in developmental and stem cell neurobiology, we have developed novel methods to generate in vitro neuronal cells of defined identity, starting from embryonic stem (ES) cells. These methods aim to produce, from pluripotent stem cells, homogenous and defined populations of neural progenitors and mature neurons of interest for clinical practice and pharmacological screens. In addition we use such systems to gain insight into the basic mechanisms of neuronal specification in health and disease, and explore their potential use in vitro for pharmaceutical research, and in vivo for rational brain repair strategies.

We have been mainly focusing on the cerebral cortex, which is the target of most neurological diseases. 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.

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.

To this aim we have focused on 3 main objectives:

1. **Development of in vitro systems for generation of clinically relevant neuronal types, from mouse and human ES and iPS cells.**
2. **Finding new genes potentially involved in neuronal specification.**
3. **Testing of the relevance of the ES/iPS systems for therapeutic graftings, in animal models of brain repair.**

1. Development of a novel model of cortical development from human pluripotent stem cells.

During brain development, it has been proposed that the forebrain emerges through an intrinsic pathway, with the anterior neural plate constituting the primitive identity of the CNS, while the more caudal parts have to be actively induced by extrinsic morphogens (Gaspard and Vanderhaeghen, 2010; Wilson and Houart, 2004). We and others recently demonstrated that this 'default' mechanism of generation of forebrain identity was also observed during neural induction from mouse ESC and human ESC (Eiraku et al., 2008; Gaspard et al., 2008; Li et al., 2009). On this basis we have described a novel *in vitro* technique for murine ES (mES) cells differentiation into cortical neurons using a chemically defined medium devoid of morphogens (Gaspard et al., 2009; Gaspard et al., 2008). In this system, mouse ES cultured in the absence of morphogens can give rise to a collection of cortical progenitors and pyramidal neurons, thereby mimicking many aspects of the regional and temporal patterning occuring during in vivo corticogenesis (reviewed in (Gaspard and Vanderhaeghen, 2010; Hansen et al., 2011).

We have now tested whether a similar in vitro corticogenesis pathway could be recapitulated using human embryonic stem (hES) cells: indeed this is an important prequisite for translational applications to follow. Importantly, although generation of cortical-like neurons has been described before from hES cells (Eiraku et al., 2008), it had remained completely unclear whether the intrinsic pathway we uncovered in mouse was conserved in human cells, and how much of the development of the human cortex could be recapitulated in vitro.

We first established a protocol for hES neural differentiation based on monolayer cultures, using the well established H9 line, in a chemically defined default medium (DDM) devoid of serum or morphogen, similar to the one used for mES cells, but supplemented with B27 to enhance neuronal survival and the Bone Morphogenic Protein (BMP) inhibitor Noggin to enhance neural induction, as previously described for hES cells (Chambers et al., 2009).

We tested different conditions to optimize a protocol for differentiation of neural progenitors, testing (i) various cell adhesion substrates, (ii) different conditions of hES cell dissociation and seeding densities. We thus set up optimized conditions of dissociation using accutase supplemented with ROCK inhibitors, and seeding using low hES cell density on matrigel substrates, followed by poly-lysin/laminin for longer term cultures. Following these conditions, up to 70% of the cells have adopted a neural fate after 15 days of differentiation, as assessed by the expression of Nestin and Pax6 at earlier stages, and also by the expression of neuronal markers like Beta-III tubulin at later time points (Espuny et al., submitted).

To identify the optimal conditions for the generation of forebrain/cortical progenitors, we then tested different combinations of morphogens or their inhibitors known to be involved in forebrain patterning, including Noggin, Wnt inhibitor Dickkopf1 (DKK1), Sonic Hedgehog and its inhibitor cyclopamine, and FGF8, followed by gene profiling using qPCR and immunofluorescence. We found that the combination of Noggin from day 0 to 8 and DKK1 from day 2 to 10 increased the expression of anterior markers like Six3 and Otx1, telencephalic markers like FOXG1 and cortical markers like Emx1/2. However, the best results for forebrain/telencephalic/cortical induction were achieved following addition of Noggin alone from day 0 to 16, resulting in the highest levels of induction of telencephalic neural identity. In these conditions, ES cell markers such as Oct4 were rapidly down-regulated (data not shown), followed by an increase in generic neural progenitor genes such as Sox1 and BLBP, and forebrain/telencephalon progenitor markers such as Emx1/2, Tbr2, or FoxG1 (**Figure 1**). In addition we explored the effects of morphogens involved in dorsal-ventral patterning of the forebrain, and found that treatments with Shh from days 10-20 resulted in a dorsal to ventral shift in regional fate, as expected. Indeed, specific genes

typical for dorsal forebrain/cortical identity like Emx1, Emx2, Pax6 and Tbr1 were decreased, while ventral forebrain markers like Nkx2.1 and Islet1 were increased. Surprisingly, we also found that the use of cyclopamine, an inhibitor of Shh, did not result in major changes in the dorso-ventral identity of the progenitors, contrary to what we found previously with mES cells. The data suggest that Shh activity is lower in the hES system, in accordance with recently published data (Li et al., 2009), and that therefore the forebrain-like progenitors generated in default conditions mainly display a dorsal identity.
Overall, our data confirm that the morphogens involved *in vivo* in forebrain development act in a similar way in hES cells, and indicate that, like for mES cells, hES cells cultured in the absence of morphogens can be efficiently differentiated into neural progenitors with a mainly forebrain-like identity, although their 'default' identity is more dorsal than ventral, presumably because of a lower Shh activity in the human system (Espuny et al. submitted).

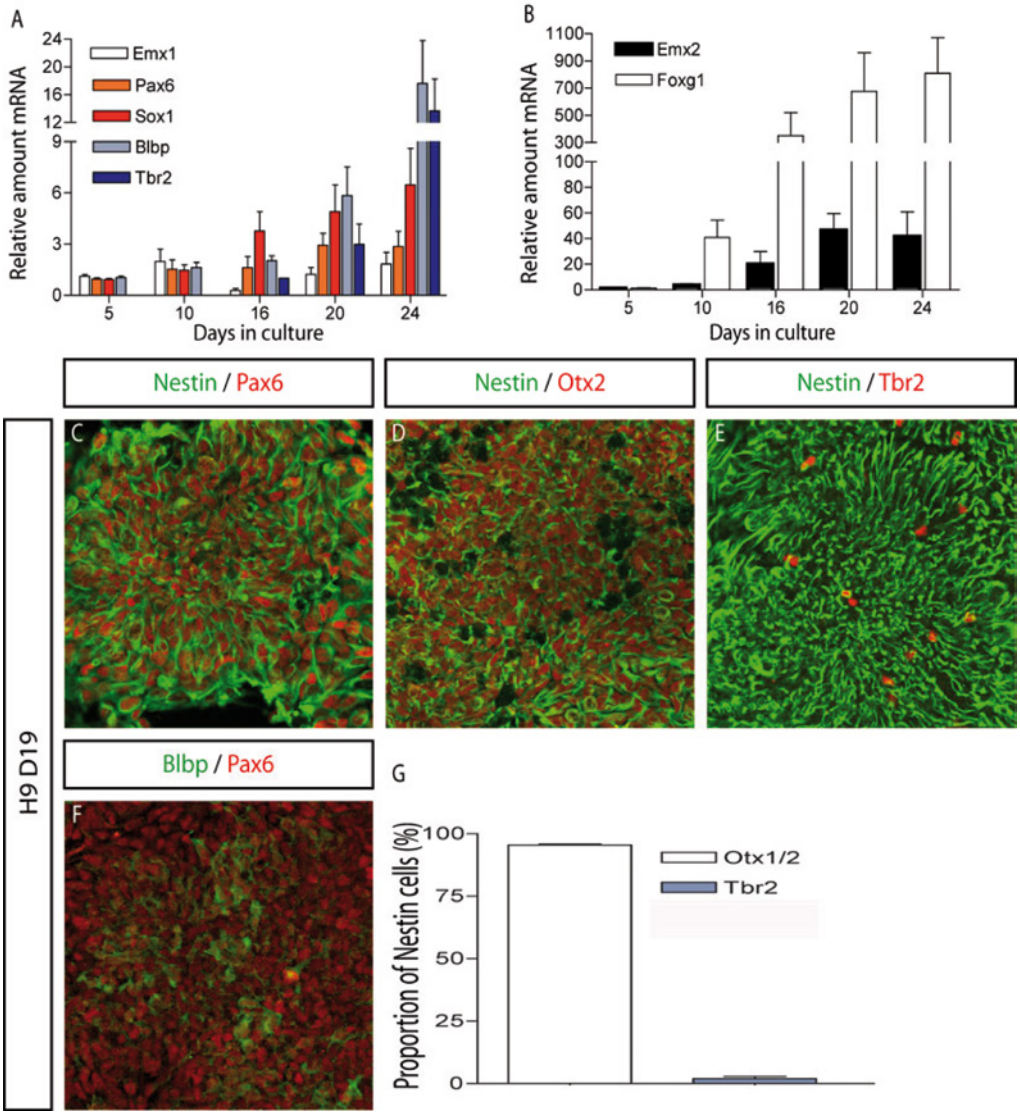

Figure 1. (A,B) qPCR analysis of the hES-derived progenitors after different time points in culture. Data are shown as relative amount of mRNA compared to the values at day 5 as value 1 +/-SEM (fold change). (C-F) Representative immunofluorescence images of day 19 cultures for the expression of Nestin/Pax6 (C), Nestin/Otx1/2 (D), Nestin/Tbr2 (E) and Blbp/Pax6 (F). (G) Quantification from 3 independent experiments of Otx1/2 and Tbr2 positive cells among the Nestin positive population of cells at day 19 +/- SEM.

While the gene expression pattern of the dorsal forebrain progenitors generated in these conditions suggest their cortical identity, the absolute definition of a cortical progenitor is its ability to generate

cortical pyramidal neurons. We thus next assessed the identity of neurons generated in these conditions, by examining cell morphology and expression of generic neuronal markers such as Beta-III tubulin and MAP2, and determining their identity using a variety of neuron subtype-specific markers, using qRTPCR and immunofluorescence (**Figure 2**).

We found that most of the neurons generated expressed markers of the glutamatergic lineage (tested by the presence of vGLUT1), while only a minority expressed markers of other neurotransmitter systems (cholinergic ChAT, monoaminergic TH, GABA-ergic vGAT) (**Figure 2**), thus, consistent with an identity of pyramidal neurons. Most of these neurons also displayed a distinctive pyramidal morphology, as expected for cortical projection neurons.

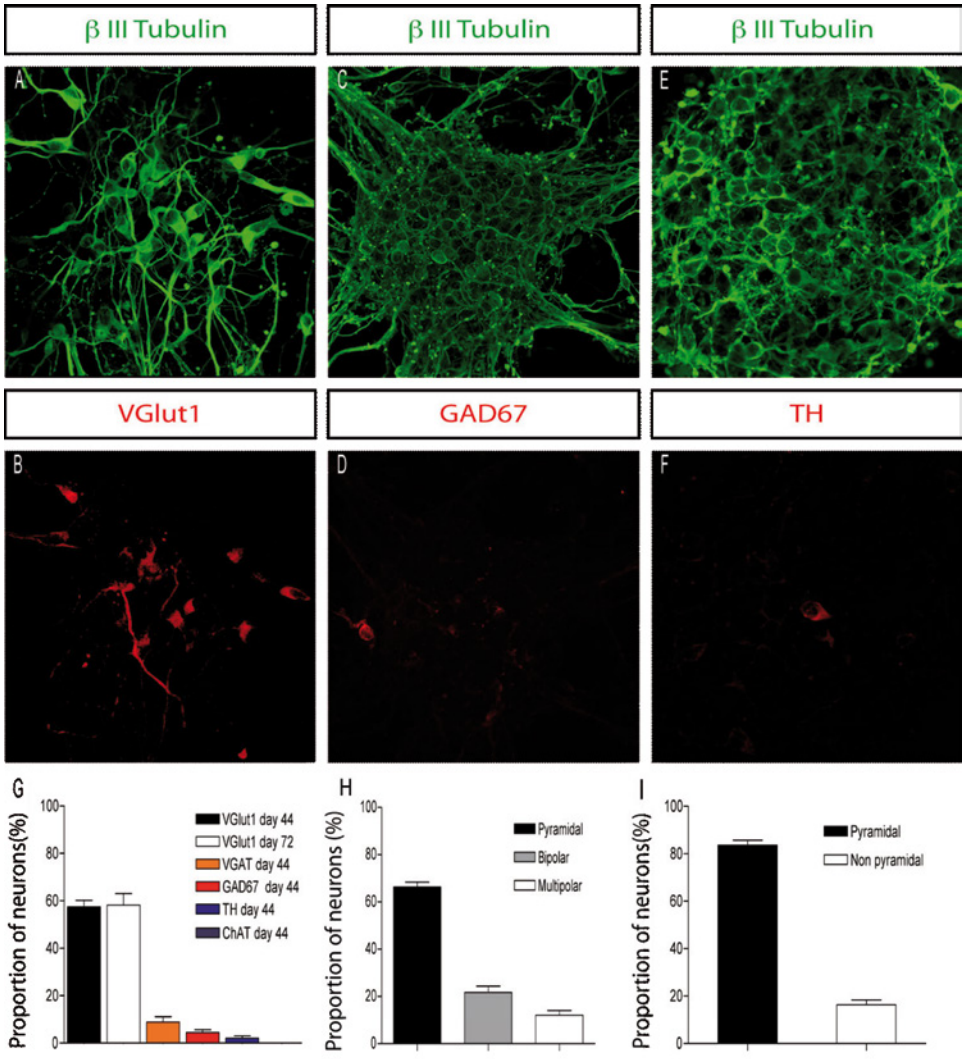

Figure 2. Human ES cells differentiate into functional mature neurons that mainly correspond to a pyramidal and glutamatergic population. (A-F) Immunofluorescence image of neurons in culture expressing beta III tubulin (in green) (A,C,E) and VGlut1 at day 72 (B), GAD67 at day 44 (D) or TH at day 44 (F) (in red). (G) Quantification of 2-3 independent experiments of the number of neurons expressing VGlut1 (days 44 and 72), VGAT (day 44), GAD67 (day 44), TH (day 44) and ChAT (day 44). (H) Proportion of neurons displaying pyramidal, bipolar or multipolar morphology (N=3 experiments). (I) Proportion of neurons displaying a PMI above the cut off of 1.2 (N=3 experiments).

Most importantly, these neurons corresponded to various subtypes of pyramidal neurons that expressed a collection of markers of cortical pyramidal identity, including markers corresponding to a variety of layer-specific cortical identity (Molyneaux et al., 2007) such as reelin/calretinin (layer I/preplate/subplate), Tbr1 (cortical identity, layer I and layer VI), FOXP2 (layer VI), CTIP2 (telencephalic identity, layer V/VI) and SATB2 (layer II/III-V) (**Figure 3**). Strikingly, like in the mouse system, these neurons appeared following

a temporal sequence highly reminiscent of the in vivo situation (**Figure 3**), but much protracted in time, as the generation of upper layer neurons only occured after two months in vitro, followed by astrocytes (as opposed to two weeks in the mouse, Gaspard et al., 2008).

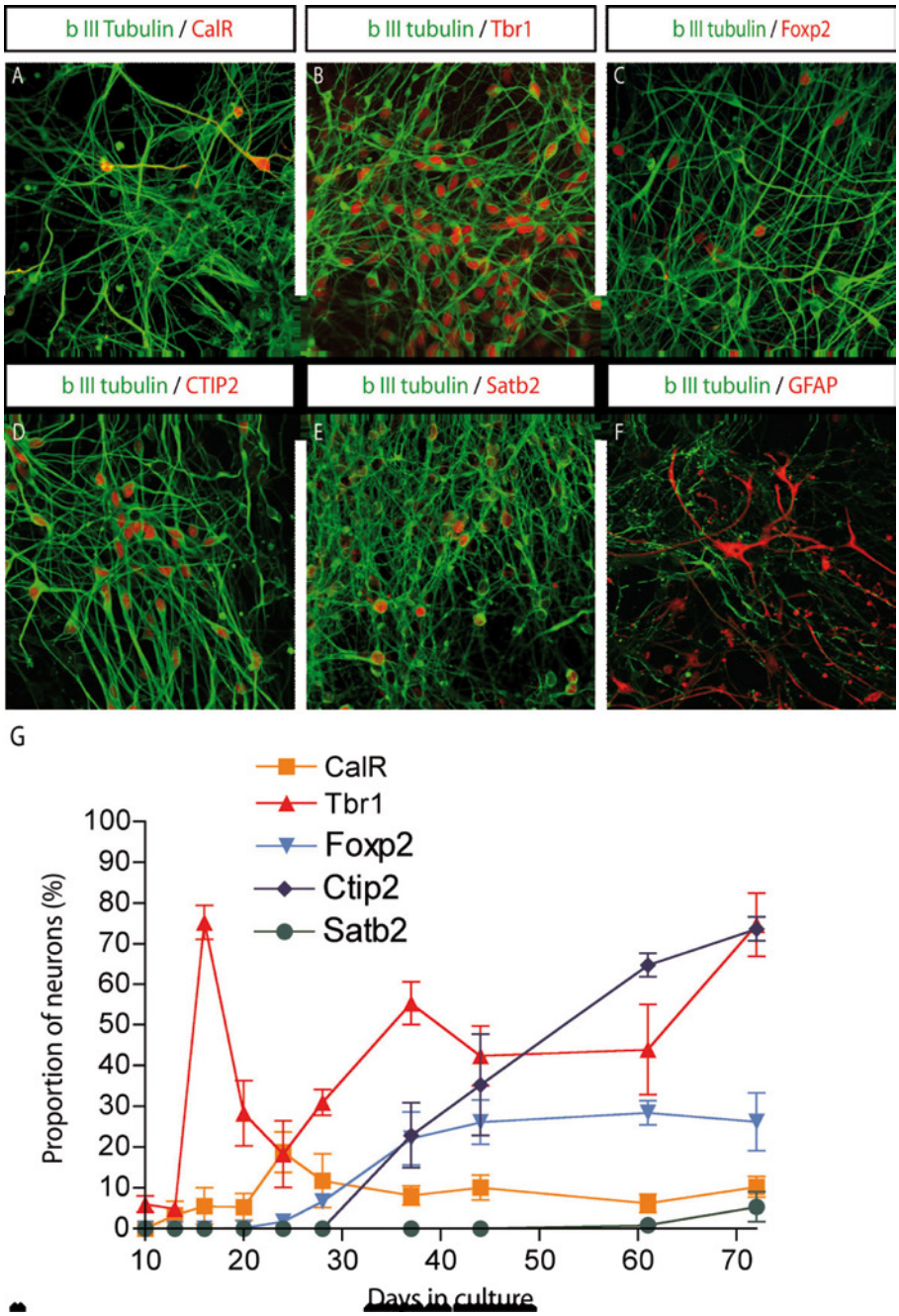

Figure 3. Neurons differentiated *in vitro* from hES cells express all the markers from the different cortical layers present *in vivo* in a time-dependent fashion.(A-F) Representative images showing the different cortical populations present at day 72 of neurons expressing beta III tubulin (in green) and CalR (subplate and layer I) (A), Tbr1 (subplate, layers I,VI) (B), Foxp2 (layers VI,V) (C), Ctip2 (layer V) (D), Satb2 (layers V, II-III) (E) and Gfap (astrocytes) (F). (G) Quantification from 3 different experiments showing the percentage of neurons expressing the different layer markers at days 10, 13, 16, 20, 24, 28, 37, 44, 61, 72 in culture +/- SEM.

In addition, we performed electrophysiological recordings (in collaboration with Dr. D. Gall, ULB) on these neurons after 40 days of differentiation, which enabled to demonstrate that they are electrically active and capable of firing spontaneous action potentials and glutamatergic synaptic currents (data not shown).

These data thus indicate that hES-derived dorsal forebrain/telencephalon progenitors mainly generate glutamatergic pyramidal neurons expressing all the molecular markers from the different cortical layers seen *in vivo*.

The efficiency and specificity of the hES differentiation into human forebrain neuronal progenitors and cortical neurons was further assessed using grafting of hES-derived cortical-like progenitors and neurons into mouse neonatal cortex, as done previously with mouse cells. For this purpose we generated an hES line ubiquitously expressing GFP as a tool for cell transplantation analysis. We grafted the hES expressing GFP-derived cortical-like cells into the NOD/SCID mouse newborn cortex and analyzed it after 1 to 6 months of age. Importantly, we found that the hES-derived neurons sent robust and specific projections similar to those of endogenous cortical neurons, including axonal projections and synapses to the right targets in the cortex, striatum, thalamus and midbrain/hindbrain, and reciprocal projections from the thalamus, most strikingly after long term grafting (**Figure 4** and data not shown).

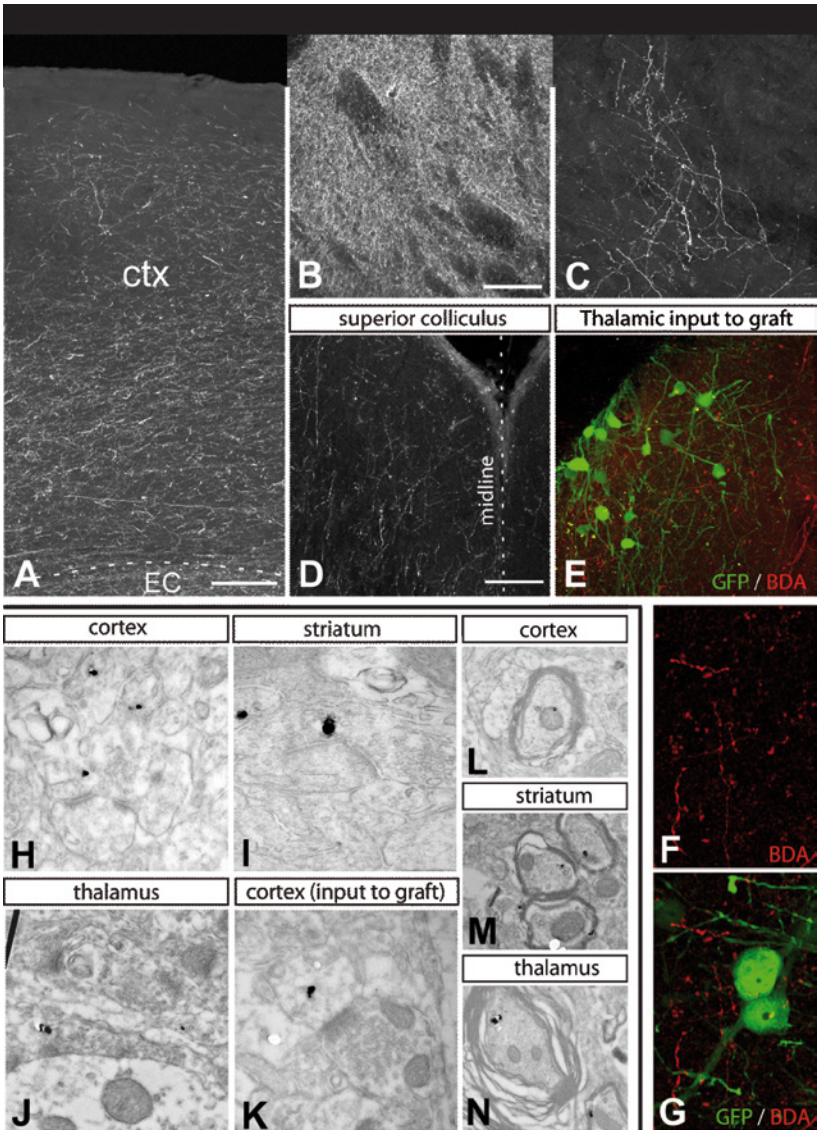

Figure 4. Following differentiation in vitro from hES cells and grafting in mouse neonatal brain, human cortical-like neurons (expressing GFP in green) can integrate in the host cortex and send dendritic and axonal projections. GFP positive fibers detected by immunofluorescence within cortical structures (ctx) external capsule (EC) (A), striatum (B), thalamus (C), and superior colliculus (D). (E) BDA positive fibers (in red) into GFP positive grafted cells (shown in green). (H-J) EM images of synaptic contacts from graft to host within the cortex (H), striatum (I), and thalamus (J) and from host to graft within the cortex (K). (L-N) EM images of GFP positive myelinated fibers within the cortex (L), striatum (M) and thalamus (N).

These important data confirm for the first time the cortical identity of human ES-derived neurons based on their morphology and pattern of connectivity with their subcortical targets.

The data described in this section above are part of a manuscript currently in revision at *Cell Stem Cell* (Espuny et al.).

Our results thus show that corticogenesis can be efficiently achieved from hES 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 mES-corticogenesis takes about three weeks to be completed, it takes more than 10 weeks starting from hES 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. Such differences are strikingly reminiscent of the properties of human corticogenesis, which takes several months in primates and is characterized by a delay in the onset of neurogenesis, which allows a larger amplification of the pool of cortical progenitors (Rakic, 2009). We now follow up on these findings by comparing the cellular and molecular properties of mES and hES-derived cortical cells, focusing on parameters that may be relevant to cortical evolution, including cell cycle properties and identity of the various classes of cortical progenitors being generated, as well as the timing of cell cycle exit and the temporal patterning of generation of neurons of distinct layer identity (Fish et al., 2008; Kriegstein et al., 2006). In order to define whether the species-specific differences observed are cell autonomous, co-cultures of mouse and human progenitors will be performed (starting from GFP-expressing hES or mES cells, already generated in the lab), followed by analysis of neurogenesis and neuronal specification. This will allow to determine whether the differences observed are linked to species-specific intrinsic properties, extrinsic cues, or both.
These experiments will allow for the first time to compare directly human and mouse corticogenesis in a comparable setup, which would be otherwise impossible to perform with *ex vivo* experiments, which could provide a highly innovative setup to study the links between development and evolution of the cerebral cortex, including genes that display human-specific patterns of gene expression. In this context, we recently completed a microarray analysis that led to the identification of several hundreds of candidate genes differentially expressed in the human fetal cortex, including several of them displaying selective patterns of evolution of their regulatory sequences (Lambert et al., 2011), which constitute a rich source of genes that could be studied functionally in the hES system.

The discovery of iPS cell technology provides unique opportunities to model and study human neurodevelopmental diseases of genetic origin, most of which remain without cure today (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). To this end, we have also started to focus on diseases for which the underlying causes remain poorly known, and where human-specific developmental mechanisms may be implicated.

As a starting point we have chosen to generate iPS models of several neurodevelopmental disorders, focusing on genes associated with syndromic primary microcephaly (MCPH) (Bond and Woods, 2006; Kaindl et al., 2010), a rare autosomal recessive condition where patients display mental retardation and a small brain size, in particular of the cerebral cortex (Bond and Woods, 2006). Mutations in several genes (MCPH1-7) have been implicated in MCPH, all of which encode centrosomal proteins, but the mechanisms by which their disruption causes defects in corticogenesis remain unclear. Importantly, at least one of these genes (ASPM, the MCPH5 gene) displays evidence of positive selection in the primate and human lineage, suggesting that it may be linked to increase of brain size during mammalian

evolution (Fish et al., 2008). The recruitement of patients was performed in collaboration with the ULB Centre of Human Genetics, following a protocol validated by the relevant Hospital and University Ethics Commissions.

During the last two years, the iPS reprogramming technology using conventional retroviral vectors allowing overexpression of Sox2/Oct4/Klf4/Myc (Takahashi et al., 2007) has been established in our laboratory, where we generated pluripotent cell lines (assessed by marker expression and tri-lineage embryoid body formation) from affected patients and unaffected controls (A. Asche and P.V., unpublished data). We thus generated already a dozen of validated iPS cell lines from three patients affected by different MCPH2-5 mutations and control patients. We also generated the first lines of patients presenting mutations in the WDR62 gene, which can cause microcephaly but also a variety of more severe cortical malformations, including gyration patterning and neuronal migration defects, associated with mental retardation and epilepsy (Bilguvar et al., 2010).
Validation of all these lines was performed using morphology screenings, gene profiling by qRTPCR and immunostainings for markers of pluripotency, as well as functional tests using embryonic bodies (**Figure 5**, and data not shown). Further validation using teratoma formation is ongoing.

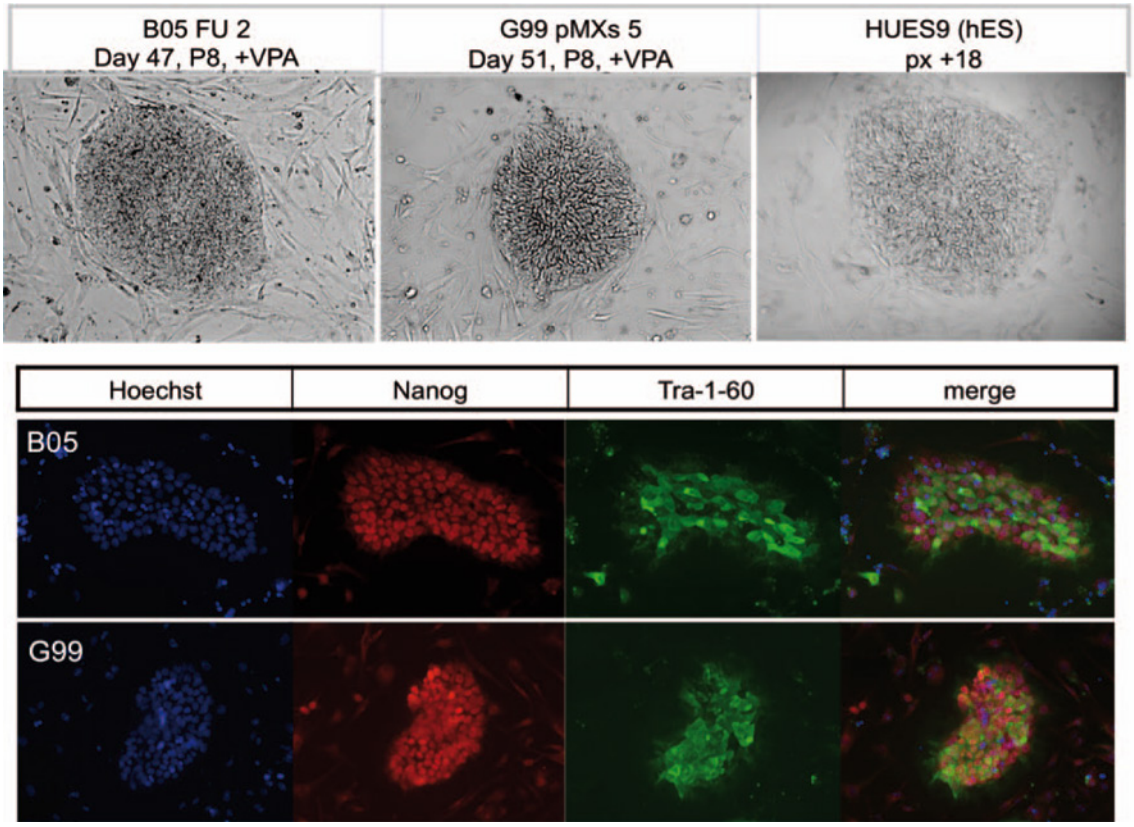

Figure 5. Generation of hiPS cells from control and MCPH patients. Upper panels illustrate the morphology of two newly generated iPS cell lines from MCPH5 patients, compared with hES HUES9 cells. Lower panels illustrate hES and pluripotency marker expression in these lines.

Using six of these validated lines, we have started to test whether a similar pathway to corticogenesis could be recapitulated from human induced pluripotent stem cells. Most importantly, our data show that our protocol of hES-corticogenesis can be applied successfully also to human iPS cells. Indeed, following the same protocol that we designed for hES cells, hiPS lines can be efficiently differentiated into neural progenitors that express similar forebrain markers, followed by generation of glutamatergic neurons expressing cortical markers (**Figure 6**, and data not shown).

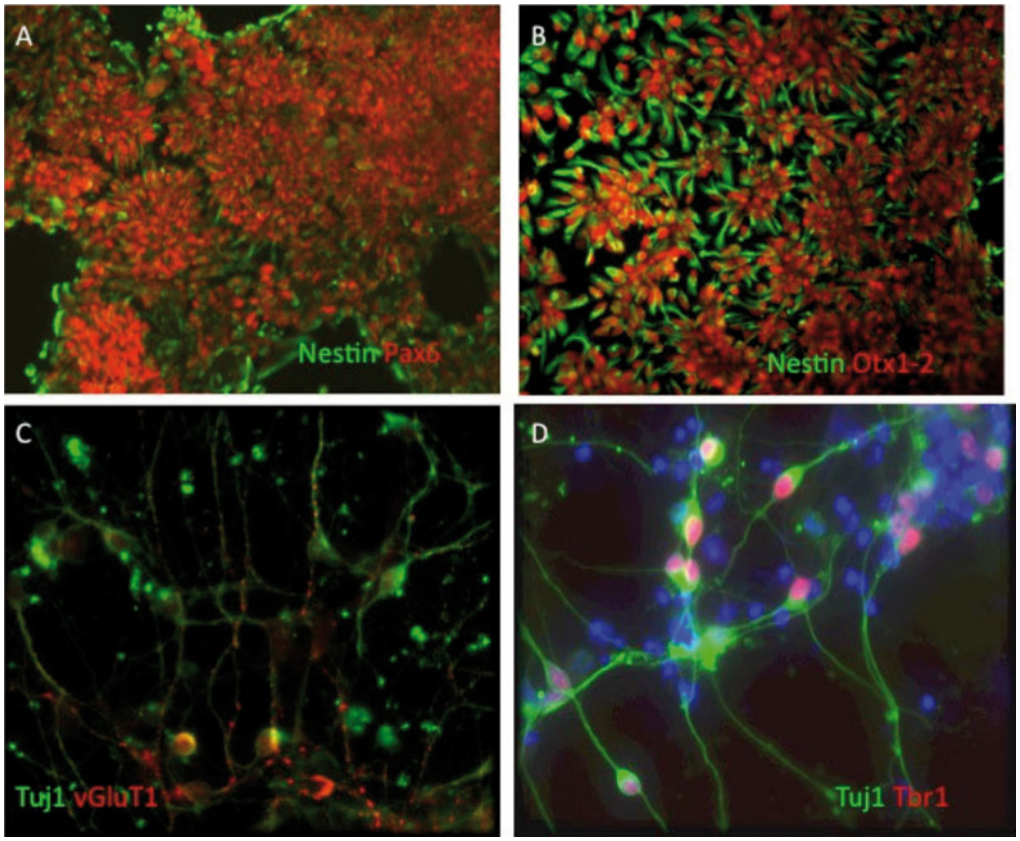

Figure 6. Generation of forebrain-like progenitors and cortical-like neurons from hiPS cells. (A-B) Following our differentiation protocol, a majority (>80%) of the cells express markers of early neural progenitors of the anterior neural plate such as Pax6 (A) and Otx1-2 (B). (C) These progenitors are competent to generate neurons that are mostly glutamatergic (C) and express cortical markers such as Tbr1 (D).

Although the long term capacity of differentiation of hiPS-derived cortical neurons still remains to be explored using grafting experiments as described for hES cell-derived neurons as well as the potential variability between cell lines as recently described for motor neuron differentiation (15), these experiments set the stage for specific differentiation from hiPS cells into cortical progenitors and neurons, including in pathological contexts.

Cortical-like progenitors derived from affected and control patients will be now examined for parameters of neurogenesis, including proliferation and survival of progenitors, patterns of symmetrical or asymmetrical division, as well as rates of neurogenesis, using markers available routinely in the laboratory. depending on the in vitro results, the phenotype of control and mutant cells will be also assessed in vivo by cortical differentiation followed by grafting experiments (as performed succesfully for hES cells), in order to characterize processes that are not possible to study in vitro, including migration (using in utero grafting, available in the lab) and longer term maturation including neuronal polarity, dendritic and axonal growth and synaptogenesis (using neonatal grafting).
This general approach should enable to determine the cellular mechanism(s) by which mutations of interest found in the studied patients can cause defects in corticogenesis. In parallel, corticogenesis from control iPS cell lines will be also tested for their functional potential in the cortical lesion grafting paradigms that we established in our first aim.
In parallel and in the longer term, we are now actively recruiting other patients presenting more complex neurodevelopmental diseases, including recently described syndromes associating changes in brain size and behavioural autistic-like phenotypes (Brunetti-Pierri et al., 2008).

2. Finding new genes involved in neuronal specification.

Our findings thus point to an intrinsic pathway of corticogenesis from ES cells, which provides an ideal tool to screen for candidate genes involved in forebrain neuron specification. Taking advantage of our observation of temporal neurogenesis based on ES cell differentiation, we currently test the functions of candidate genes on temporal neurogenesis. We use an inducible system of gene expression dependent on tetracycline (Kyba et al., 2002) obtained in collaboration with Dr Kyba (Texas SW U.). This system enables to generate easily ES cell lines that allow a precise Tetracyclin-dependent induction of candidate genes. More than 20 candidate genes are currently being tested in that frame, in particular transcription factors. We already identified several interesting transcription factors, Bcl6, Mixl1, DMRT5 and Tbr2, which have interesting and unexpected effects on the fate of neural progenitors and neurons, which we are now exploring further.

Bcl6 is a striking example of such a gene, which was previously identified as an oncogene and transcriptional repressor in B cells, but for which very little is know regarding brain development, although it was shown to be expressed to be expressed in the developing cortex (Bedogni et al., 2010). Using our inducible gain of function system, we found that Bcl6 overexpression induces potent neurogenesis, in particular of Tbr1 positive neurons (**Figure 7a**). This proneural effect is associated with rapid repression of Hes5, suggesting a direct interaction between Bcl6 and the Notch pathway, which is currently under investigation.

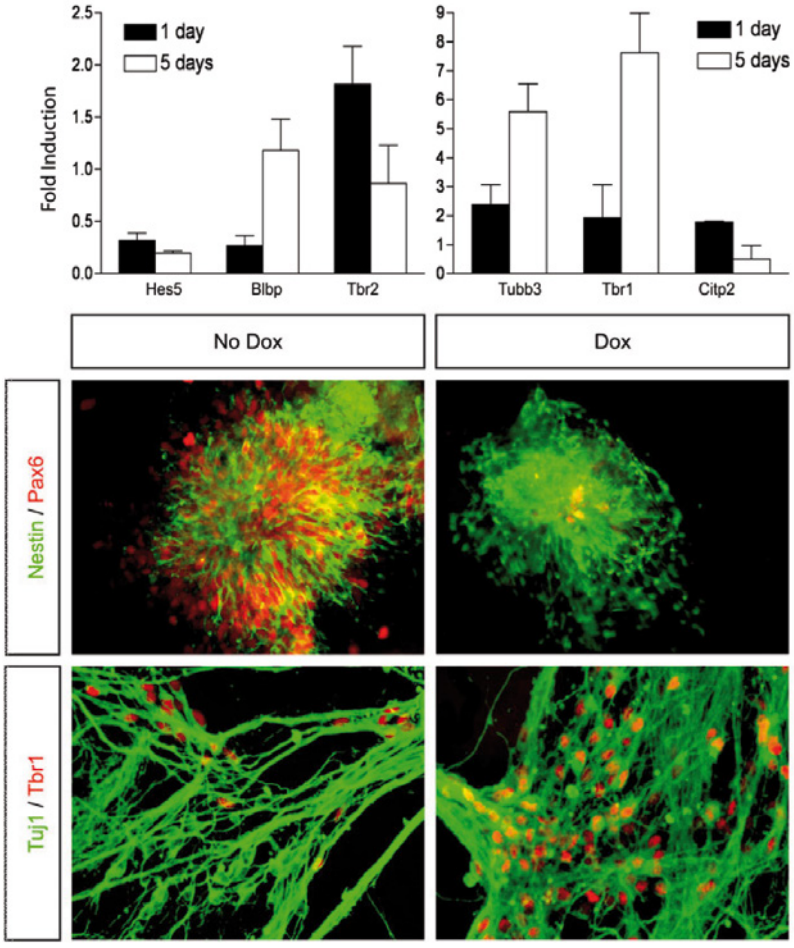

Figure 7a. Gain of function experiments of Bcl6 in vitro. Following overexpression of Bcl6 (with Dox treatment using a tetracyclin dependent transgene expression), Hes5 is rapidly downregulated in cortical progenitors, which are then massively converted to Tbr1+ cortical neurons.

These data identify Bcl6 as a potential novel transcriptional regulator of cortical neurogenesis. To test further this hypothesis, we started the analysis of Bcl6 knock-out mice (obtained from Dr. De la Favera, Columbia U.). Strikingly, this in vivo approach supports the in vitro data, as loss of function of Bcl6 results in early defects in the generation of cortical neurons, associated with decrease of cell cycle exit and generation of intermediate progenitors (**Figure 7b**, and data not shown).

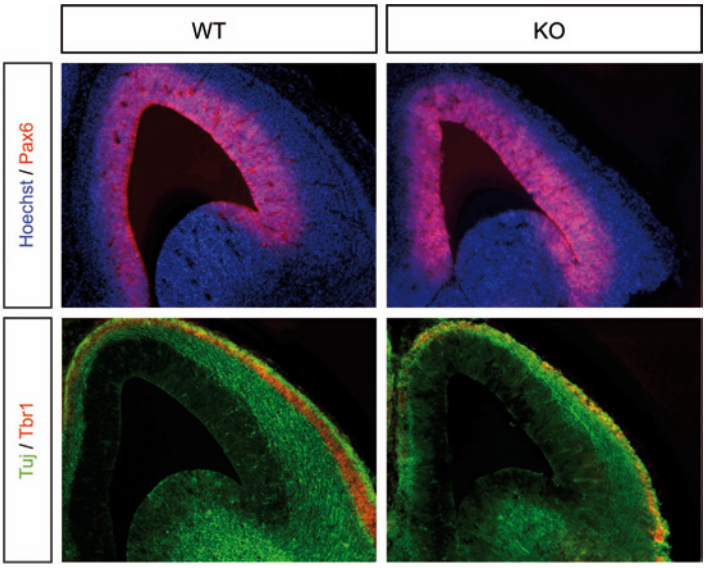

Figure 7b. Loss of function of Bcl6 in vivo (E12 cortex). In Bcl6 ko mice, the generation of Tbr1+ cortical neurons is drastically reduced at early stages of corticogenesis.

The data described in this section above are part of a manuscript currently in revision at Nature (Tiberi et al.).

We follow up on these promizing data to identify the molecular mechanism by which Bcl6 controls cortical neurogenesis, looking at specific interactions with the Notch pathway, and in the longer term looking at its transcriptional targets using gene profiling in the in vitro gain of function model and ko mice. In parallel, the study of other transcription factors positively identified in our ES system-based screen will be further investigated, in vitro and in vivo.

3. Exploring the use of ES-derived neurons for brain repair.

We previously found that mES-derived cortical neurons can be grafted efficiently into neonatal mouse cortex, where they can display patterns of axonal projections that are similar to thos of endogenous cortical neurons (Gaspard et al., 2008). While these data constitute strong evidence for the cortical identity of ES-derived neurons, they raise the possibility that in vitro corticogenesis from ES cells could be used also in the perspective of neuronal replacement following cortical damage. Indeed, it was recently demonstrated that native embryonic cortical neurons, when grafted in the lesioned adult cortex, can reestablish subcortical projections that were altered by the cortical lesion (Gaillard et al., 2007).

We have thus started to explore the relevance of our model for cell replacement following cortical lesions in the adult mouse brain (Michelsen et al., submitted). To achieve this, we first implemented a relatively simple experimental setup (**Figure 8a**), similar to the one previously described for native embryonic neurons. 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, ES-derived cortical progenitors and neurons (generated following our chemically defined protocol that has been now tailored for many different mES cell lines (Gaspard et al., 2009)) were grafted at the same site of the lesion. In order to track the identity and projections of the grafted cells, we used Tau-GFP lines where GFP is expressed under the control of the neuronal Tau promoter. The identity and projections of the grafts were then monitored one to two months following grafting.

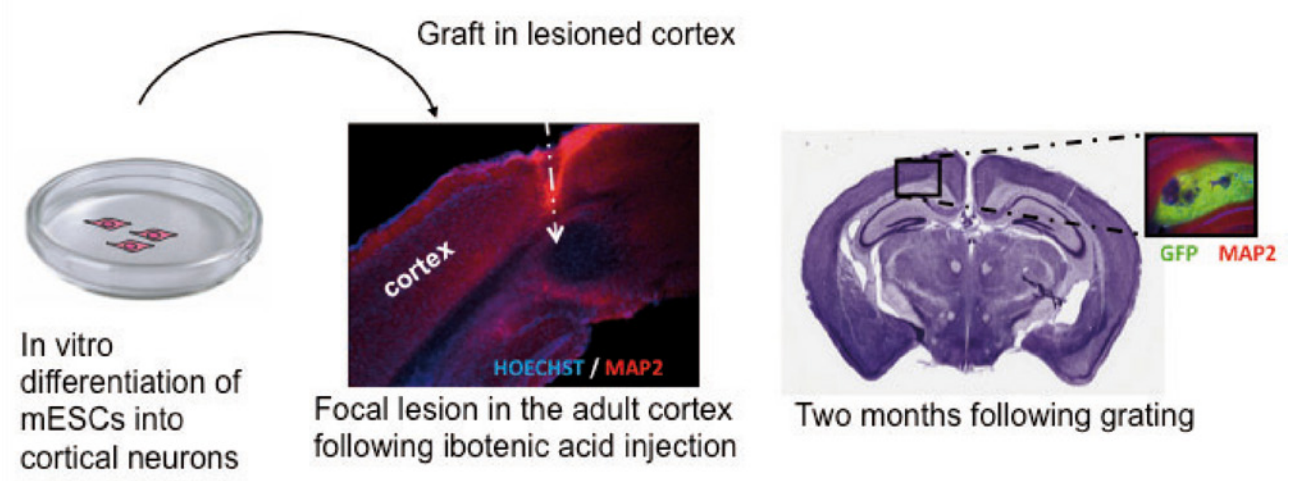

Figure 8a. Schematic view of the experimental setup used to test ES-derived cortical neurons in adult lesioned cortex. mES-derived cortical neurons were grafted three days following a focal neurotoxic cortical lesion. Properties of the grafted cells were analyzed one to two months following grafting. GFP/MAP2-stained ES-derived cortical neurons can be observed two months following grafting (inset).

Analysis of grafted animals (N=55 cases) indicated that most of them (80%) contained a graft, consisting mainly of differentiated neurons (expressing Tau/GFP and MAP2) (**Figure 7**), expressing markers of cortical pyramidal identity (Tbr1/FoxP2/CTIP2; data not shown). Most importantly, inspection of the rest of the brain one month after grafting revealed in 40% of the cases far-reaching graft-derived axonal growth, following specific paths (corpus callosum, internal and external capsule, cerebral peduncles) and reaching specific targets of endogenous cortical neurons, including ipsi- and contraleral cortex, and subcortical structures such as striatum, thalamus, and midbrain/hindbrain nuclei (**Figure 8b**). In addition, electron microscopy combined with immunogold labelling for GFP on brains two month following grafting, demonstrated that the transplant-derived axons can be myelinated and make synapses with the host, including at the level of subcortical targets in striatum, thalamus and midbrain (**Figure 8b**, and data not shown).

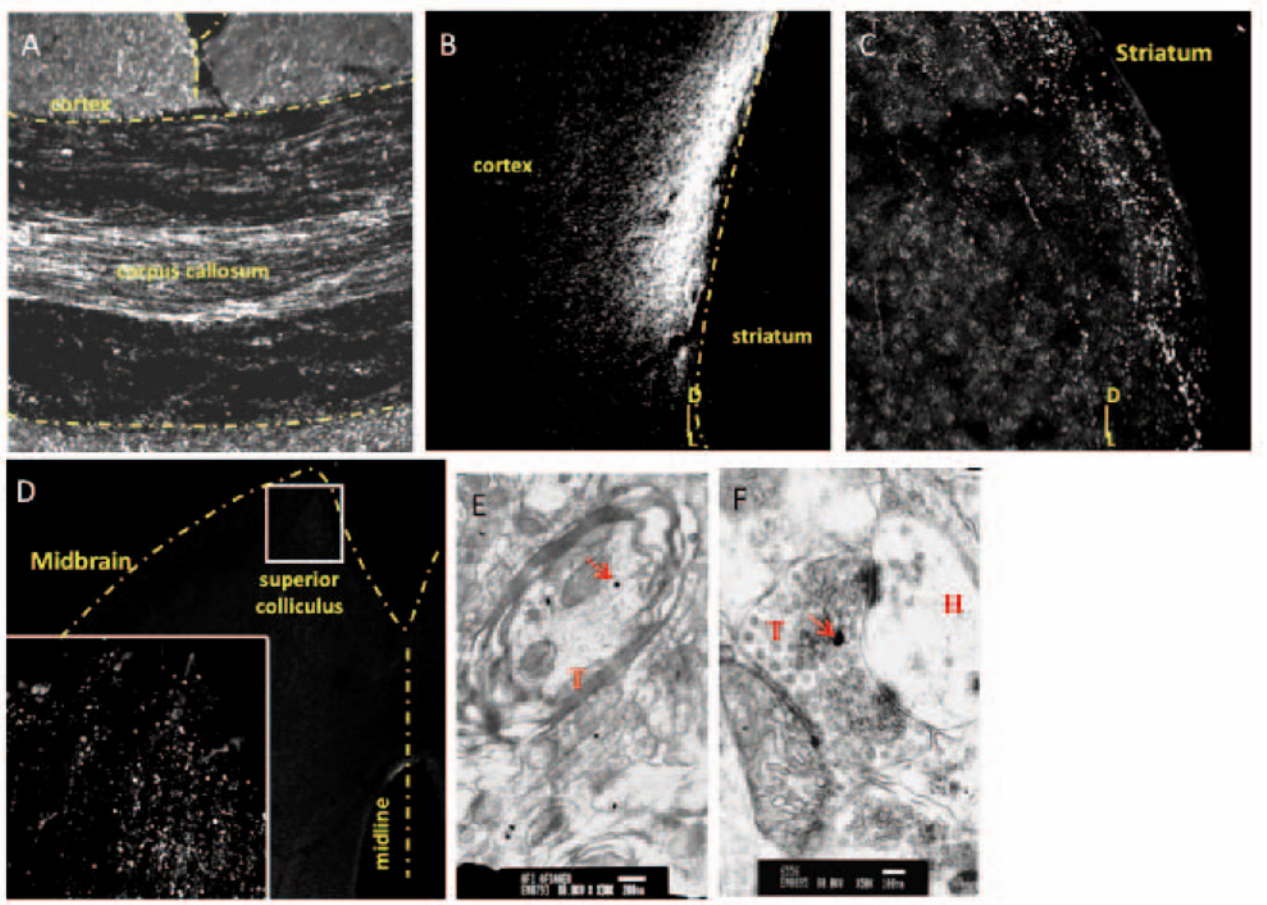

Figure 8b. Patterns of axonal growth and connectivity of ES-derived cortical neurons following grafting in adult lesioned cerebral cortex. (A-D) GFP+ fibers detected one month post-grafting, in corpus callosum (A), cortex (B), striatum (C) and midbrain (D). (E,F) Electron microscopy analysis using GFP immunogold labeling (red arrows) demonstrates (E) myelination of the transplanted neuron axons (T), and (F) transplant (T) to host (H) synapses to subcortical targets (striatum).

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 (**Figure 8c**).

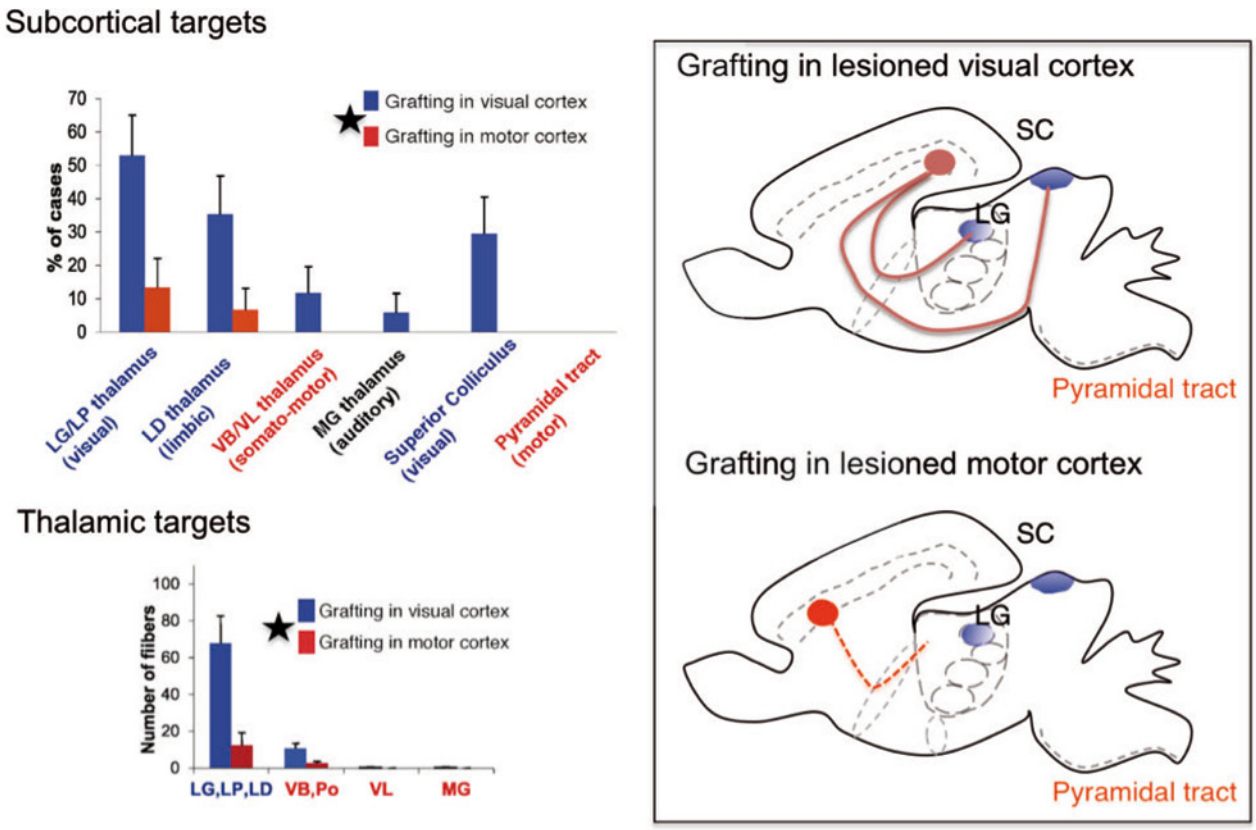

Figure 8c. Patterns of axonal growth and connectivity of ES-derived cortical neurons following grafting in adult lesioned cerebral cortex. Following grafting in lesioned visual cortex, axons specifically innervate visual targets, thus reestablishing specifically the damaged pathways. Following grafting in motor cortex, very few subcortical projections are observed, pointing to the importance of a match between the identity of the lesioned neurons and the grafted neurons.

Collectively, these data demonstrate for the first time that ES-derived cortical neurons can establish specific patterns of connectivity within the adult brain following a cortical lesion, which constitutes an important first step towards the rational study of pluripotent stem cell-derived neurons in brain repair strategies targeting the cortex.

The data described in this section above are part of a manuscript currently submitted at *Cell Stem Cell* (Michelsen et al.).

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