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

Verslag – Rapport – Bericht – Report

2013

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

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Geneeskundige Stichting Koningin Elisabeth
2013
Inleiding verslag activiteiten van de GSKE – FMRE

Het jaar 2013 is het einde van de kredieten die werden toegekend voor een periode van 3 jaar (2011-2013) aan 13 door het wetenschappelijk comité van de Geneeskundige Stichting Koningin Elisabeth geselecteerde ploegen. (zie het verslag van 2012).

Een oproep voor projecten werd, via de decanen van de faculteiten geneeskunde en wetenschappen, naar alle universiteiten van het land gelanceerd.

De projecten moesten binnen zijn vóór 15 juni. Het wetenschappelijk comité heeft 96 neurowetenschappelijke projecten geanalyseerd, op het gebied van de geneeskunde, de moleculaire biologie en de psychologie.

Deze zeer moeilijke taak was eind november afgelopen. Eerst werd gewerkt per elektronische post om vervolgens af te sluiten met een voltallige vergadering. Dit heeft geleid naar een selectie van 14 teams op basis van 40.000 euro per team en dit gedurende drie jaar, indien de financiële toestand dit toelaat. Dit brengt dus het totale bedrag op 560.000 euro. Dit classement werd bekrachtigd door de Raad van Bestuur op 05 december. Het resultaat van de beraadslaging kon op Sinterklaas 2013 aan de teams overgemaakt worden.

Op 16 mei heeft de overhandiging van de wetenschappelijk prijzen van de Stichting plaats gevonden in het Koninlijk Paleis te Brussel. Deze prijzen werden, in overeenstemming met het reglement, door het wetenschappelijk comité toegekend aan de geselecteerde teams. De selectie is gebaseerd op de verslagen van de activiteiten van het afgelopen jaar. De Raad van Bestuur heeft het voorstel van het wetenschappelijk comité bekrachtigd.

De prijs Solvay aan Dr. Pierre Vanderhaeghen (ULB), de prijs van Gysel de Meise aan Prof. dr. S.N. Schiffmann (ULB), de prijs Janine et Jacques Delruelle aan Prof. dr. Danny Huylebroeck (KU Leuven) en de prijs CBC Bank aan Prof. dr. Geert van Loo (UGent)

Ter gelegenheid van deze zitting, hebben wij genoten van de uiteenzetting van Professor Jan De Maere, internationaal erkend expert op het gebied van kunstwerken, vooral de Vlaamse schilderkunst. Een originele uiteenzetting over “Het meesterwerk en het brein, neurowetenschappen en emoties”. Dit onderwerp heeft heelwat nieuwsgierigheid en interesse opgewekt, zowel door de kwaliteit van de uiteenzetting als door de voorgestelde illustraties.

Het is altijd met veel plezier en met grote vreugde dat ik de leden van de Raad van Bestuur kan danken voor hun dynamische en toegewijde steun en hun interesse in het werk van de Stichting. Mijn oprechte dank gaat in het bijzonder naar Prinses Astrid, onze Erevoorzitster, die ondanks Haar zeer drukke agenda alle werkzaamheden van de Stichting met enthousiasme volgt.

Prof. em. dr. Baron de Barsy,
wetenschappelijk directeur
Brussel, december 2013

Fondation Médicale Reine Elisabeth
2013
Introduction rapport d'activités de la FMRE - GSKE

L'année 2013 a vu la fin des crédits attribués pour une période de 3 ans (2011-2013) à 13 équipes sélectionnées par le comité scientifique de la Fondation Médicale Reine Elisabeth (cfr rapport 2012).

Un appel à projets a été lancé dans toutes les universités du pays, par l'intermédiaire des doyens des facultés de médecine et de sciences.

Les projets, devaient être rentrés pour le 15 juin. Le comité scientifique a été amené à analyser 96 projets de neurosciences, couvrant la médecine, la biologie moléculaire et la psychologie.

Cette tâche très difficile s'est terminée en fin novembre, le conseil ayant travaillé d'abord par courrier électronique puis clôturé par un réunion plénière, a permis un classement de 14 équipes à raison de 40.000 euros par équipe et par an durant trois ans, si la situation financière le permet. Ceci amenait donc la somme globale à 560.000 euros. Ce classement a été entériné par le conseil d'administration en date du 5 décembre. Les équipes ont donc pu être averties des délibérations le jour de la Saint Nicolas 2013.

Le 16 mai a eu lieu au Palais Royal de Bruxelles, la remise des prix de la Fondation, prix attribués aux équipes sélectionnées par le comité scientifique en accord avec le règlement. Ce sont les rapports d'activités de l'année écoulée qui ont servi de base à la sélection. Le conseil d'administration a entériné la proposition du comité scientifique.

Le prix Solvay au Dr. Pierre Vanderhaeghen (ULB), le prix van Gysel de Meise au Prof. dr. S.N. Schiffmann (ULB), le prix Janine et Jacques Delruelle au Prof. dr. Danny Huylebroeck (KU Leuven) et le prix CBC banque au Prof. dr. Geert van Loo (UGent)

A l'occasion de cette séance, nous avons pu bénéficier de l'exposé du Professeur Jan De Maere, expert internationalement reconnu dans le domaine des œuvres d'art, surtout en peinture flamande. Il nous a fait un exposé original sur "Le chef-d'œuvre et le cerveau, neurosciences et émotion". Ce sujet a suscité beaucoup de curiosité et d'intérêt, tant par la qualité de l'exposé que par les illustrations présentées.

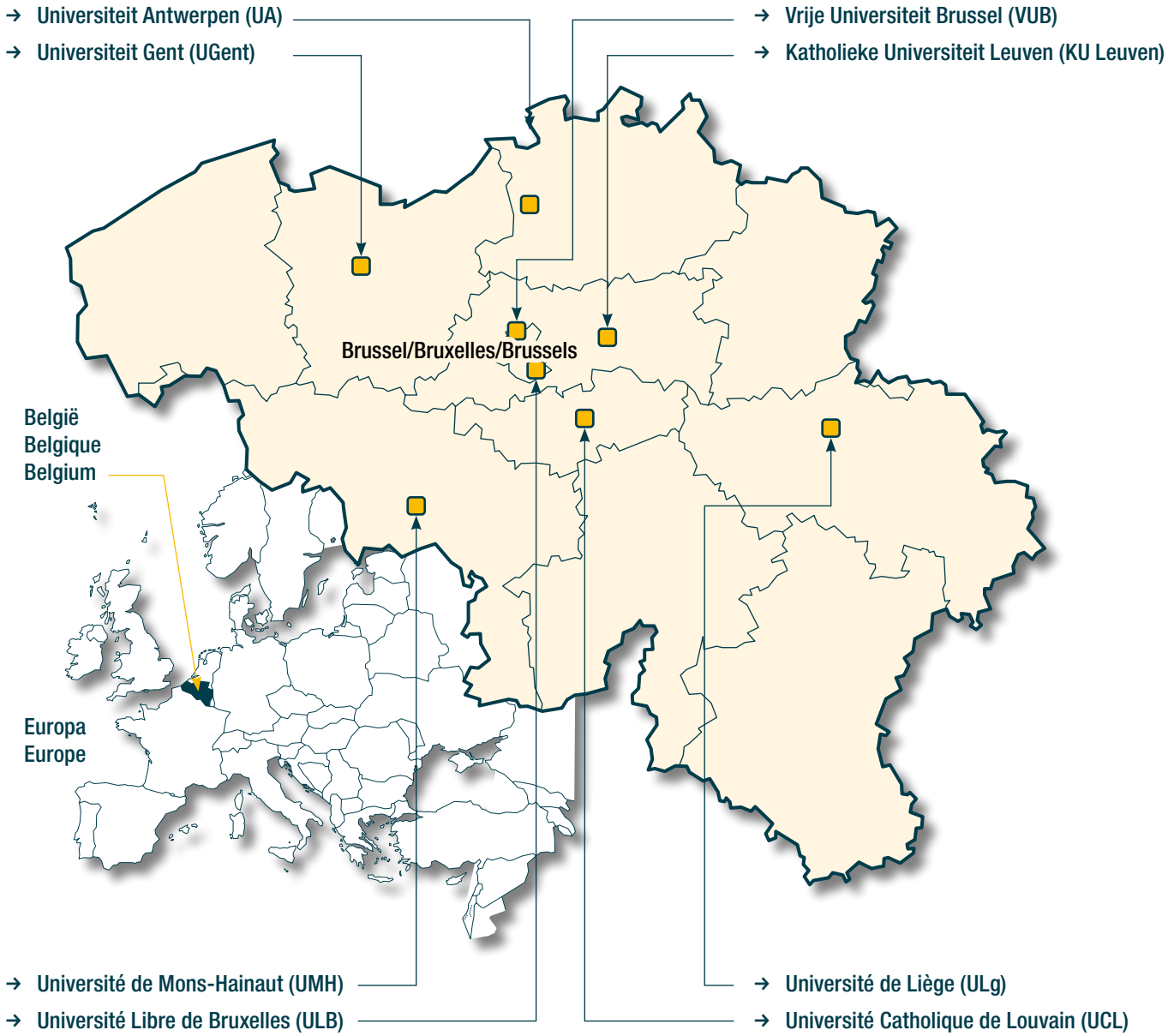
C'est toujours un énorme plaisir et une grande joie de remercier les membres du conseil d'administration pour leur soutien dynamique et compétent et leur investissement dans le travail de la Fondation. Mes remerciements chaleureux s'adressent tout particulièrement à la Princesse Astrid, notre présidente d'honneur, qui, malgré son agenda très chargé, suit avec enthousiasme tous les travaux de la Fondation.

Prof. em. dr. Baron de Barsy,
directeur scientifique
Bruxelles, 30 décembre 2013

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

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

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



Onderzoeksprogramma's gefinancierd door de G.S.K.E. - Programma 2011-2013

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

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

KU Leuven

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

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

UA

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

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

UCL

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

UGent

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

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

ULB

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

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

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

ULg

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

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

VUB

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

Final reports of the university research groups, supported by the Queen Elisabeth Medical Fondation in collaboration with the following professors and doctors (2013)

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

β-actin is the form traditionally associated with cell migration but more and more a role for transcriptional regulation becomes evident (Visa and Percipalle, 2010). Primary embryonic fibroblasts (MEFs) isolated from the knock-out mouse have increased expression of γ- and α-smooth muscle actin and (re) differentiate to a myofibroblast-like phenotype. These cells display strongly impaired cell migration and increased adhesion. In addition we observed that the Rho – Rho-kinase (ROCK) pathway is over-activated and the MEFs have sustained transforming growth factor β (TGF-β) activity. Inhibiting ROCK or myosin contractility restores cell migration, strongly indicating that altered signaling is generating the impaired migration, rather than lack of actin polymerization capacity (Tondeleir *et al*. 2012). Collectively, our results point to an important role of actin in cell and organ differentiation via its essential nuclear function (Tondeleir *et al*. 2012, Tondeleir *et al*., 2013) and not via its traditional role as engine for cell motility.

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

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

We exploit the *Actb*$^{-/-}$ mouse model. Consistent with defective MEF migration, we observed in β-actin KO embryos phenotypes that can be attributed to impaired cell migration: e.g. aberrant vascularization (requiring endothelial cell migration) and, as documented in the 2011 report, an amazing lack of neurofilament staining at E9.5. We also showed that trunk neural crest cell (NCC) where specified but they displayed a migration defect in the embryo. In addition *Actb*$^{-/-}$ NCC had a different morphology of wild type (*Actb*$^{+/+}$) NCC in neural explants and remained much more together in a cell sheet. We could only partially restore this phenotype using ROCK inhibition, suggesting that not only the Rho - ROCK pathway was affected but also different pathways were deregulated. In view of the increased cell cell adhesion we hypothesized cadherin signaling could be altered. This was part of the 2012 report. In conjuction with mapping Crap 1 expression to further document the NCC migration defect in embryos, we investigated N-cadherin and Cadherin 11expression in neural tubes at different embryonic stages and actin cytoskeletal morphology in neural crest cells (see report 2012). These experiments were further complemented as described below leading to new insights in the ontogeny of neural crest cells (Tondeleir et al., 2014).

The neural crest cell migration defect observed in *Actb*$^{-/-}$ embryos could either be due to reduced migration capacity (as suggested by the qualitative experiment with the ROCK inhibitor) or due to the fact that the migrating *Actb*$^{-/-}$ cells undergo apoptosis. We explored both possibilities. We quantified the migration ability of neural crest cell using explants treated or not with ROCK-inhibitor and determined the migration index of the cell sheet. In the absence of inhibitor the migration of the of the *Actb*$^{-/-}$ NCCs was severely impaired. Treating with ROCK-inhibitor alleviated the migration defect (despite not restoring cell morphology, see report 2012). This is consistent with previous observation on *Actb*$^{-/-}$ MEFs (see introduction, Tondeleir *et al.*, 2012) and suggests that the Rho-ROCK pathway is over activated in *Actb*$^{-/-}$ NCCs and that other actin isoforms drive the restored cell migration. However, cells remained more together likely by altered cadherin function (see below).

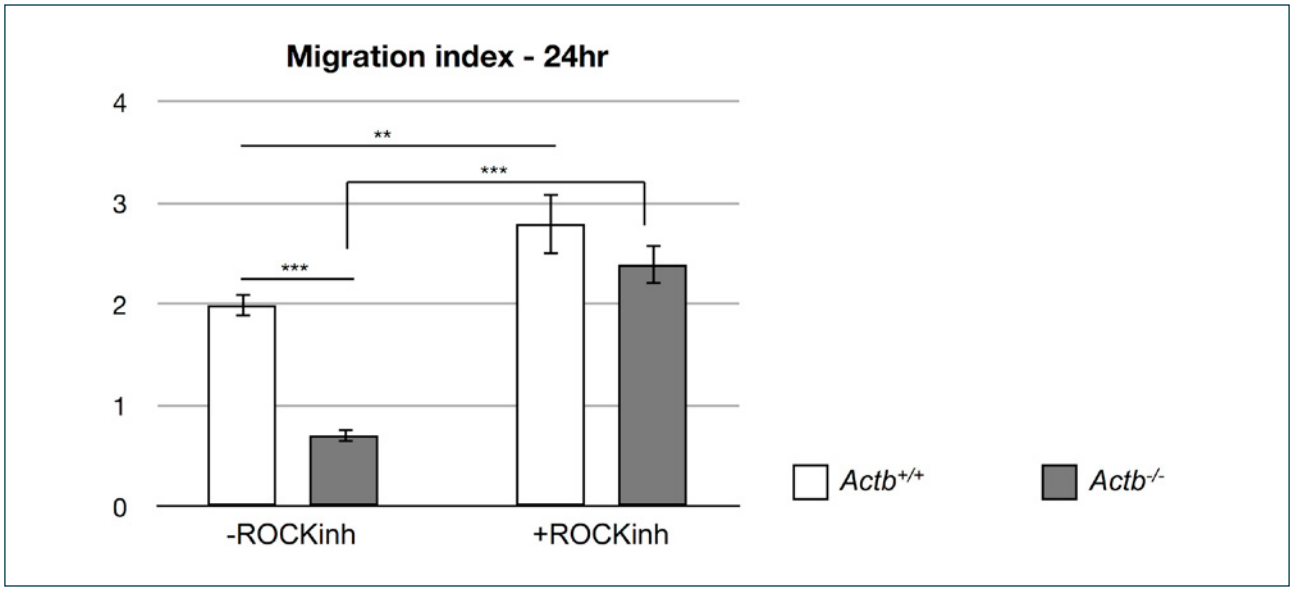

Figure 1: the migration index of Actb-/- NCCs versus WT NCCs as determined by neural explant assays after 24hrs. This was determined with (right) and without (left) adding ROCK-inhibitor at time zero.

Cell proliferation was not affected in *Actb*$^{-/-}$ embryos as evidenced by phospho-Histone 3 staining. However we provided evidence for increased apoptosis using TUNEL analysis on sections and this was also the case for migratory *Actb*$^{-/-}$ NCCs (see Fig 2). Thus in addition to a bona fide migration defect the reduced number of NCCs reaching their distant destinations is also due to increased cell death.

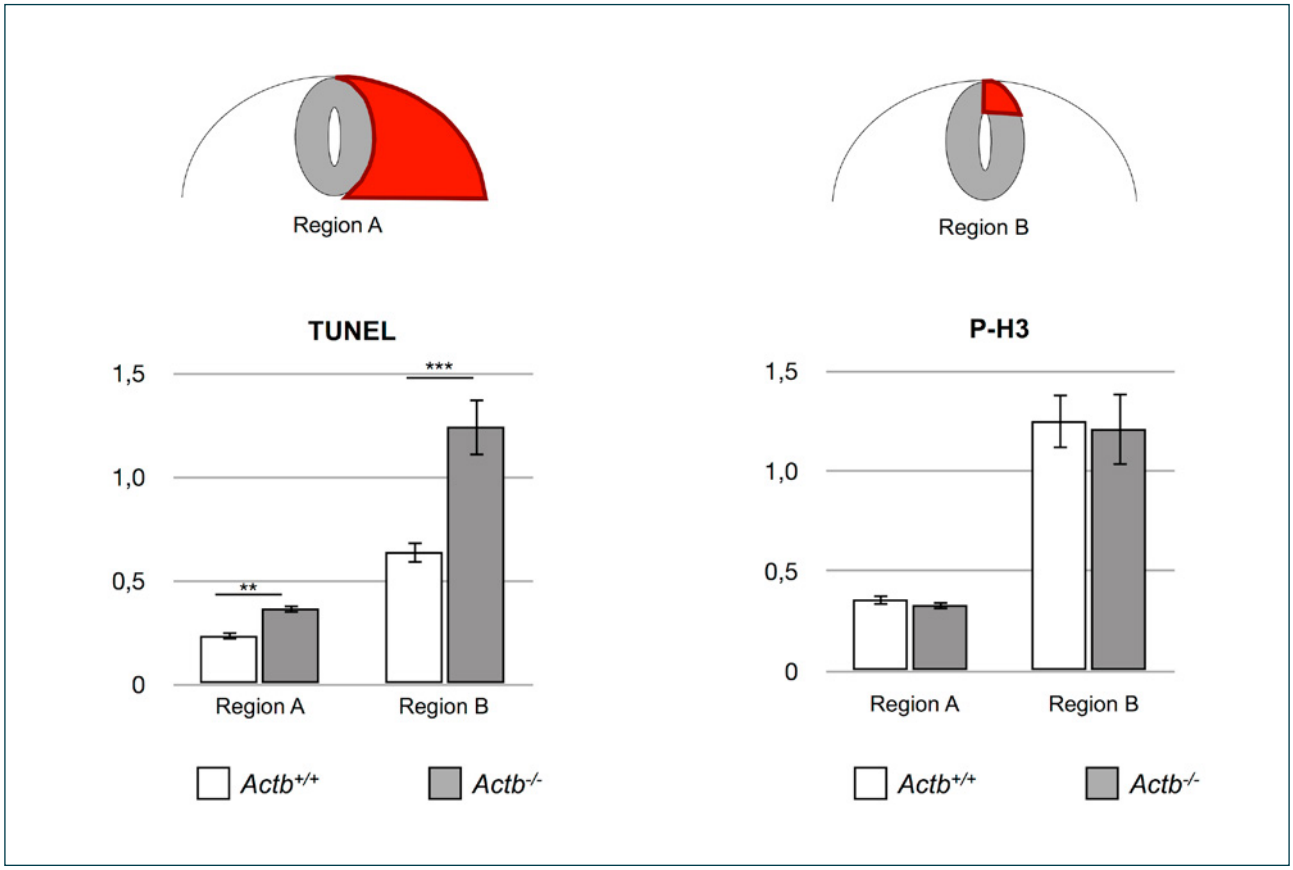

Figure 2: The migratory *Actb*$^{-/-}$ NCCs display increased apoptosis. Section of embryos where costained with p75 (a marker for NCCs and with Br-dUTP (TUNEL reagent) or an anti p-H3 antibody. Both the migratory region (A) as the region where NCCs originate (B) where imaged.

Given the observed phenotype of the NCC in explants (behaviour as a cell sheet) and given the importance of cadherins in cell-cell interactions and the connection with actin cytoskeleton we paid attention to cadherin switching which occurs during NCC epithelial mesenchymal transition (EMT) prior to delamination in other animal or cell models (Acloque *et al.*, 2009, Wheelock and Johnson, 2003). In the 2012 report we showed aberrant cadherin expression in neural crest cells of Actb$^{-/-}$ embryos. Briefly N-cadherin and cadherin 11 expression was normal in sections of E8.5 embryos but at E9.5 cadherin 11 levels in *Actb*$^{-/-}$ neural crest where much reduced and most migrating neural crest cells were organized as clusters rather than streams.

Since the Rho ROCK pathway is connected to cadherin signaling (Theveneau and Mayor, 2012) we further explored the effect of the ROCK-inhibitor on cadherin expression ex vitro. In WT explants the majority of the N-cadherin is membrane bound. *Actb*$^{-/-}$ NCCs do express N-cadherin but this remains cytosolic. E-cadherin shows a punctuate staining in WT-cells and is virtually absent from *Actb*$^{-/-}$ NCCs. Interestingly, *Actb*$^{-/-}$ explants treated with ROCK-inhibitor, 48hr prior to staining, had clear cadherin 11 staining (Figure 3). Thus the increased migratory capacity of ROCK-inhibitor treated NCCs in explants correlates well with the increased expression of this type II cadherin. Treatment with ROCK-inhibitor diminished membrane bound N-cadherin localization and increased cytosolic and perinuclear staining.

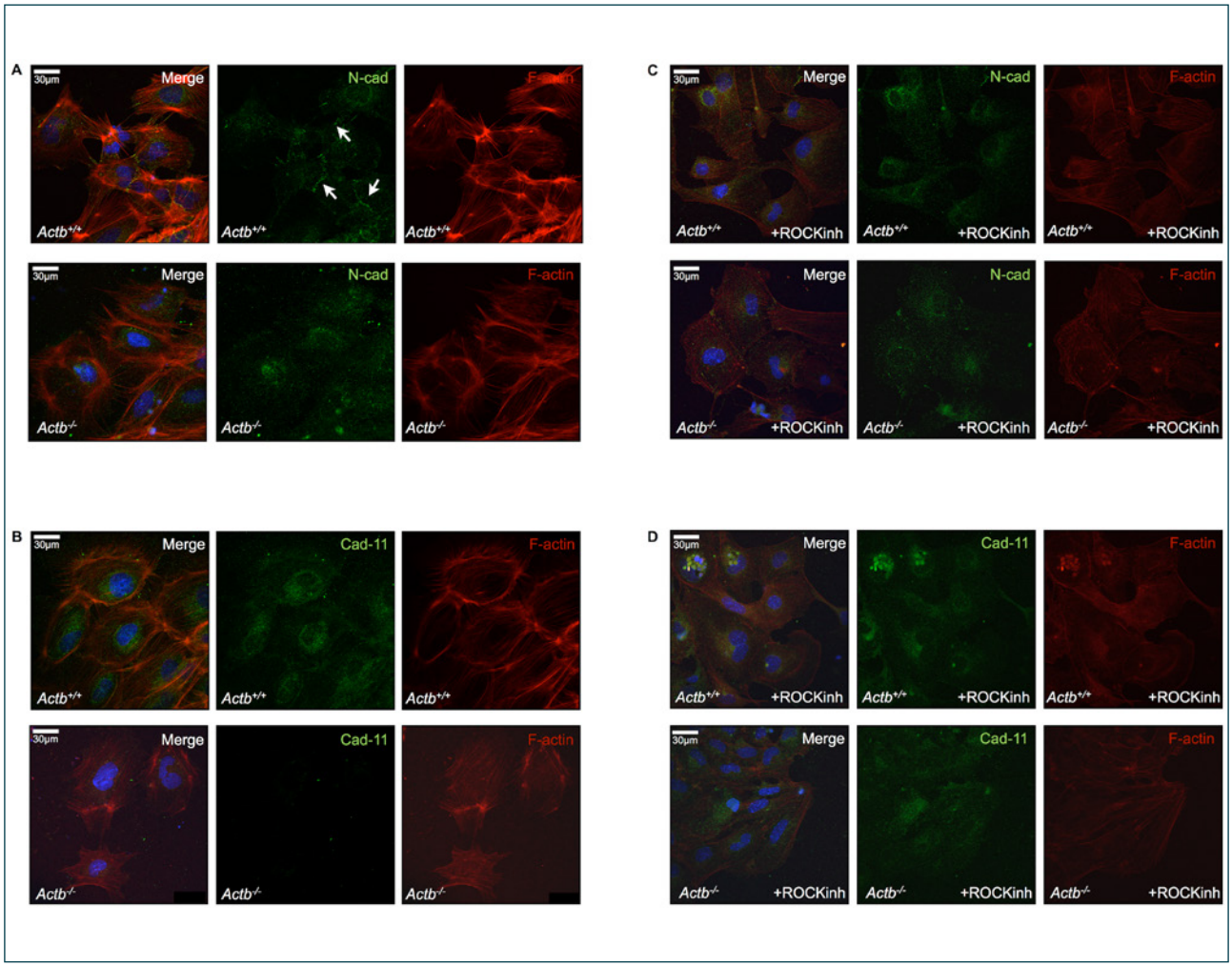

Figure 3: Cadherin staing (green) and filamentous actin (F-actin) staining (red) of WT Actb$^{+/+}$ and Actb$^{-/-}$ NCCs in explants treated (right) or not (left) with ROCK-inhibitor. DAPI (blue) was used to stain the nuclei.

We note that N-cadherin expression and cadherin 11 expression and the associated switch from a type I to a type II cadherin has not been described in WT-mice at the investigated stages, neither was the absence of E-cadherin expression in NCCs at E9.5 (data not shown). Also the influence on subcellular localization of N-cadherin in function of ROCK activity has not yet been documented Although these were important control experiments they also revealed these molecules and pathways contribute to normal development, in particular cadherin switching during trunk neural crest cell migration positioning it downstream of Rho.

In summary β-actin is required for proper mouse neural crest cell ontogeny. The observed NCC migration defect is likely due to several causes: improper EMT and altered post-transcriptional signaling that in the NCC cells resulting in aberrant cadherin expression. These data are consistent with an upstream regulatory role of β-actin suggesting again that its nuclear function, and not its cytoskeletal function, is the more important one. This also in view that other actin isoforms can drive cell migration in NCCs if the Rho-ROCK pathway is inhibited.

The work described here and from the previous reports is now published (Tondeleir et al., 2014), part of the cadherin work resulted in a Master Thesis (Rivka Noelander 2012) and the gobal study was part of the PhD doctoral thesis of Davina Tondeleir that was successfully defended (March 29, 2012).

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Final report of the research group of

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

State of the art and summary of the research program

Memory formation and cognitive processes rely on activity-dependent synaptic plasticity. Synaptic inputs dictate the time, place and amount of protein synthesis necessary for the single synapses. Dysregulation of these mechanisms leads to spine dysmorphogenesis (Fiala et al., 2002) and to a variety of pathological conditions including the most common form of inherited mental retardation due to the absence or mutation of a single protein, FMRP (Bagni et al., 2012). FMRP is involved in multiple steps of neuronal messenger RNA metabolism such as transport, stability and local translation. We have shown that FMRP, together with its cytoplasmic interactor CYFIP1, controls, in an activity dependent manner, the synthesis of key proteins at synapses, which are impaired in a mouse model for FXS.

The protein CYFIP1 has a critical role in human brain function/s. Deletions and/or duplications of CYFIP1 have been associated to Autism, Schizophrenia and Epilepsy (Bittel et al., 2006; Chai et al., 2003; Doornbos et al., 2009; Tam et al., 2010; van der Zwaag et al., 2010; von der Lippe et al., 2010). Using a mouse model (Cyfip1 KO) and other model systems (Bozdagi et al., 2012; Schenck et al., 2003; Star et al., 2002) it has been shown that CYFIP1 is involved in the formation of synaptic processes. Therefore unbalances in CYFIP1 interactive network/s might result in neuronal dysfunctions as dendritic spines dysgenesis: a common cellular phenotype observed in patients with Fragile X Syndrome and other mental disorders (Fiala et al., 2002).

In the proposed project we plan to: 1) Identify and characterize the CYFIP1 complexes in different neuronal compartments, 2) Investigate whether the Rho GTPase Rac1, a CYFIP1 interactor, regulates CYFIP1-dependent protein synthesis, 3) Study the physiological regulation of FMRP and CYFIP1 upon synaptic activation.

This project will shed light into mental retardation, Autism and possibly schizophrenia.

Results

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

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

35 novel CYFIP1 interacting proteins, in addition to other 4 already identified in other studies, including RNA-binding proteins implicated in mRNA metabolism and actin cytoskeleton regulators. Intriguingly, the composition of the complexes changes according to the their neuronal subcellular localization, with more RNA-binding proteins identified in the whole-cell CYFIP1 complexes, and more cytoskeletal proteins identified in synaptoneurosomes (Fig. 1).

A literature search on disease involvement of the genes in question revealed that 25 out of the 40 proteins that bind CYFIP1 are encoded by genes associated with ID, ASD, ADHD, schizophrenia, major depressive disorder, and Alzheimer's disease (Fig. 1). In addition, a gene-based analysis interrogating for association with schizophrenia based on the meta-analytic p values obtained by the largest schizophrenia genome-wide association study to date (Ripke et al., 2011) (9,394 cases and 12,462 controls) revealed that 8 out of 36 tested autosomal genes of the CYFIP1 interactome had a nominally significant p value (<0.05) for association with schizophrenia. This significantly exceeds the expectation (1.8 genes) under the null hypothesis of no association (one-sided Fisher's exact test, p = 0.042), although the polygenic nature of schizophrenia should be considered. One gene, FAM120A, was significantly associated with schizophrenia (p = 0.00064) after Bonferroni correction for testing 36 genes. In summary, 25 proteins out of 40 identified in our CYFIP1 interactome are encoded by genes involved in diseases: 26% are associated with schizophrenia, 19% with ASD, and 10% with ID. These observations suggest that CYFIP1 and its interaction partners are linked to pathways that, if impaired, can be associated with intellectual disabilities and psychiatric disorders. These data have now been published in the journal Neuron (De Rubeis et al., 2013).

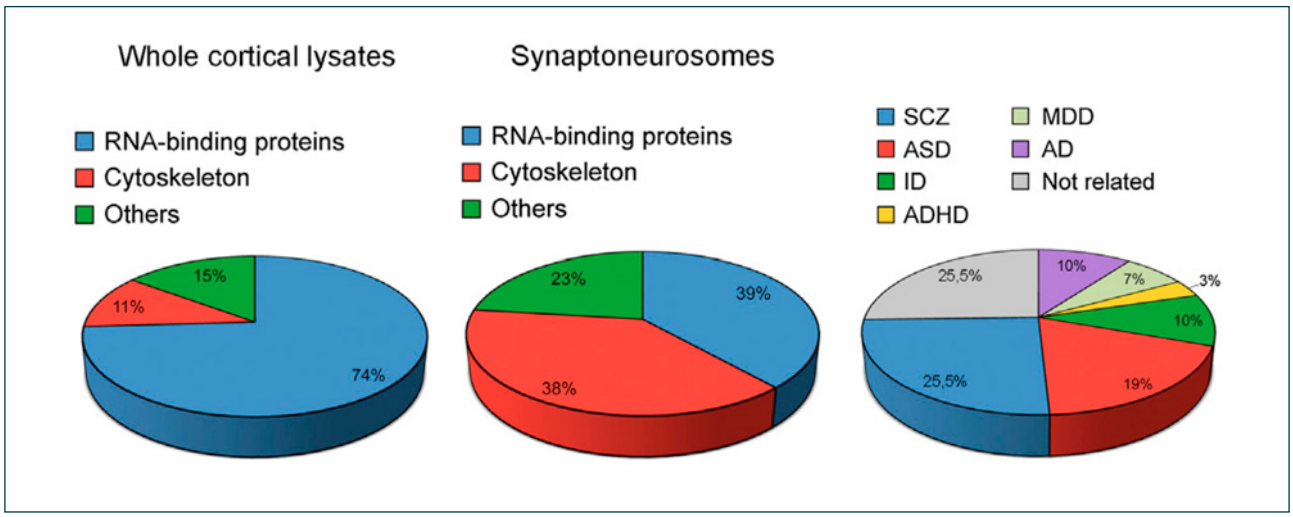

Figure 1. The CYFIP1 Interactome in mouse Cortex and Synapses and Its Relevance for neuropsychiatric disorders (Left Panel) CYFIP1 interactome, as revealed by MS of the proteins coimmunoprecipitating with CYFIP1 in cortical whole-cell lysate. IP with rabbit IgGs was used as a negative control; n = 3. (Middle Panel) Outcome of MS analysis of the proteins coimmunoprecipitating with CYFIP1 in cortical synaptoneurosomes. IPs with rabbit IgGs were used as negative control; n = 6. (Right Panel) Many CYFIP1 interactors are linked to neurological diseases. Indicated are the percentages of genes related to intellectual disability (ID), autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), schizophrenia (SCZ), major depressive disorder (MDD), and Alzheimer's disease (AD) (the diagram is approximate, as some genes are related to more than one disease). From De Rubeis et al., 2013.

During the last year of support we have mainly focused on the validation of the CYFIP1 interactome on *post mortem* brain tissues from patients with schizophrenia, depression and bipolar disorder and unaffected controls from the Stanley Consortium (https://www.stanleygenomics.org/collect.html). Our data show that FMRP (Fragile X Mental Retardation Protein, a key interactor of CYFIP1 is dysregulated in Schizophrenia and Bipolar disorder patients. Specifically, we have analyzed post-mortem brain samples from the prefrontal and the cingulate cortex. Patients with depression (n = 15), bipolar disorder

(n = 15), and schizophrenia (n = 15) were compared by Western blotting against control individuals (n = 15), (Achsel et al., in preparation). We analyzed the levels of FMRP, CYFIP1, and a number of interacting proteins and found some correlations in certain brain areas indicating that the CYFIP1 complexes are disturbed in psychiatric diseases.

We would like to validate these findings in blood from patients with such complex disorders hoping to **identify new genes/markers for such complex disorders.**

Aim 2: *CYFIP1-mediated regulation of actin cytoskeleton remodeling and local protein synthesis.*
Achievement 2: The CYFIP1 interactome revealed the existence of two complexes: 1) CYFIP1-FMRP-eIF4E, implicated in translational control and 2) the WAVE Regulatory Complex (WRC), involved in remodeling of the actin cytoskeleton. Therefore, we hypothesized that the activation of the small GTPase Rac1, which acts upstream of the WRC, might release CYFIP1 from eIF4E and relocating it on the WRC. We were able to prove that those events occur as response to synaptic activity. We have now directly studied the dynamics of CYFIP1-eIF4E interaction in synaptoneurosomes stimulated with BDNF in presence or absence of Rac1 inhibitors and showed that the effect of this inhibitor on activity-induced local protein synthesis is dependent on CYFIP1 and/or FMRP: siRNA knock-down of Cyfip1, genetic depletion of Fmrp, and pharmacological inhibition of Rac1 with NSC23766 all abolish the 2.5 fold induction of Arc that is normally observed upon BDNF stimulation (Fig. 2, see lower left panel for quantification).

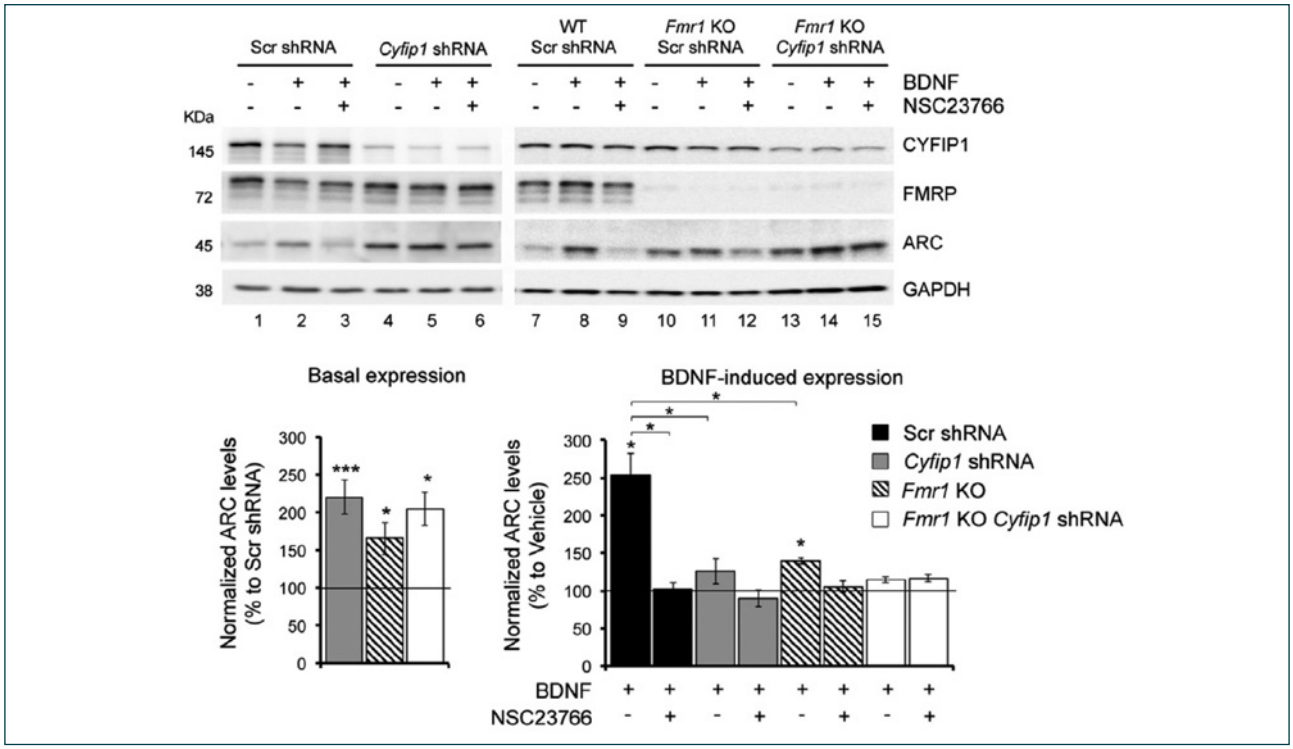

Figure 2. Rac1 Regulates CYFIP1 and FMRP-Dependent mRNA Translation. Synthesis of ARC is altered in Cyfip1 knockdown, Fmr1 KO, and Fmr1 KO/Cyfip1 knockdown neurons. Upper panel: immunoblot of cortical neurons stimulated with BDNF (100 ng/ml for 30 min) +/– NSC23766 (200 mM for 10 min pretreatment). Lanes 1–3, scrambled shRNA; lanes 4–6, Cyfip1 shRNA, lanes 7–9, scrambled shRNA in WT neurons; lanes 10–12, scrambled shRNA in Fmr1 KO neurons; lanes 13–15, Cyfip1 shRNA in Fmr1 KO neurons. Lower right panels: basal expression of CYFIP1, FMRP, and ARC levels in vehicle-treated neurons. Protein levels were normalized to GAPDH and shown as percentage of scrambled shRNA. Grey, Cyfip1 shRNA; gray stripes, scrambled shRNA in Fmr1 KO; white, Cyfip1 shRNA in Fmr1 KO (n = 6, one-way ANOVA with Holm's post hoc correction, *p < 0.05, ***p < 0.001). Lower right panel: activity-induced ARC expression. ARC levels after BDNF +/_ NSC23766 expressed as percentage to vehicletreated neurons. Black, scrambled shRNA; gray, Cyfip1 shRNA; gray stripes, scrambled shRNA in Fmr1 KO; white, Cyfip1 shRNA in Fmr1 KO (n = 6, two-way ANOVA with Holm's post hoc correction, *p < 0.05). Bars represent mean ± SEM. From De Rubeis et al., 2013.

As a result of this dysregulation, dendritic spines are more immature (De Rubeis et al., 2013, see also Fig. 3), a phenotype that is observed in many intellectual disabilities and that is thought to be crucial for the disorders. To understand which of the two complexes is responsible for the aberrant spine morphology, we aimed at uncoupling the two complexes and studying their contribution to protein translation and actin polymerization. For this purpose, we designed specific CYFIP1 mutants impairing the interactions with either eIF4E or the WRC component NCKAP1. To reduce the CYFIP1-eIF4E interaction, we used a mutant replacing Lys743 with a Glu (mutant E), which has been shown to reduce the interaction with eIF4E (Napoli et al., 2008). To interfere with the CYFIP1-NCKAP1 complex, we studied the large surface of interaction between the two proteins (Chen et al., 2010), and found two hydrophobic patches on CYFIP1 that fit to corresponding sites on NCKAP1. The second patch (Ala1003–Ile1010) shows a higher complementarity to NCKAP1 and was predicted as an essential binding site for NCKAP1. We therefore designed two mutants: mutant D, lacking the C-terminal domain that harbors the hydrophobic patch (aa 922–1251), and mutant H, in which the eight hydrophobic residues were replaced by glycines. To promote the incorporation of the exogenous proteins into functional complexes, we silenced the endogenous Cyfip1 with siRNAs directed against its 3'UTR. Both mutant D and mutant H lost their affinity for NCKAP1 and consequently for WAVE1, but not for eIF4E, whereas the interaction with eIF4E was largely decreased with mutant E, leaving unaffected the binding to NCKAP1 and WAVE1 (De Rubeis et al., 2013).

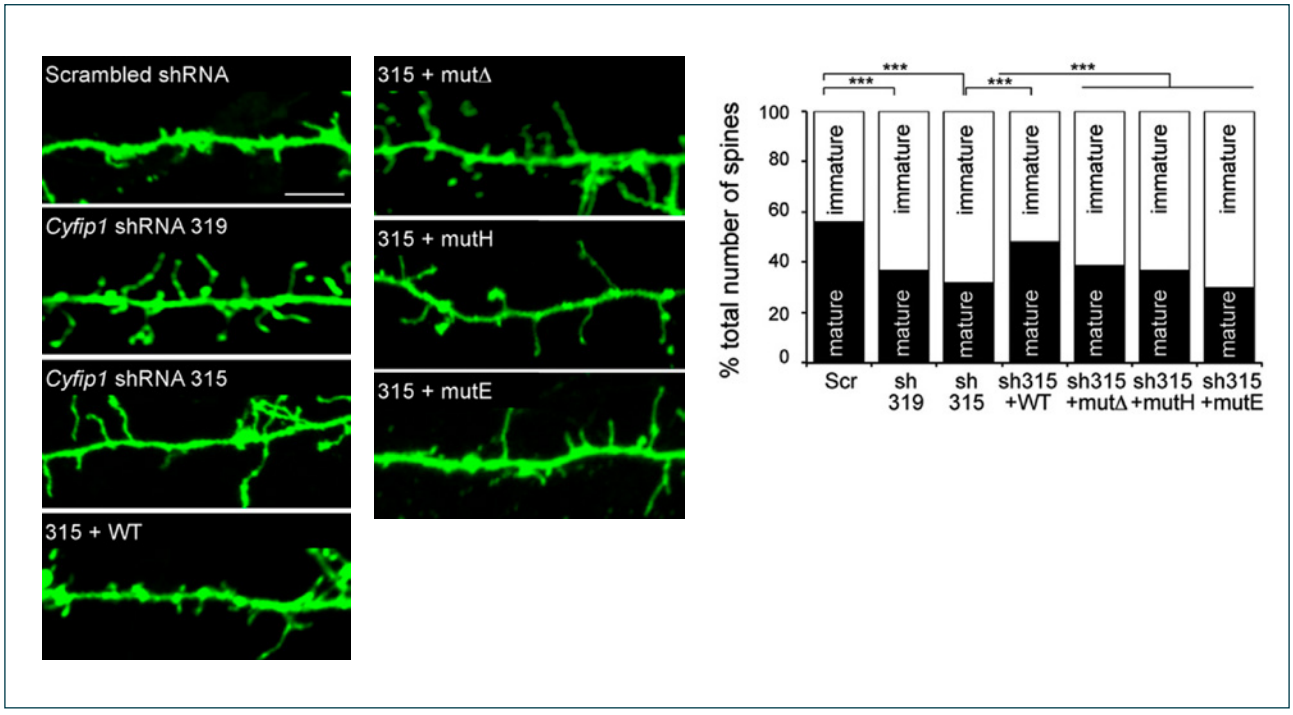

Figure 3. Dendritic spines are altered in cultured cortical neurons silenced for Cyfip1. (Left panels) Outline of dendritic shafts from DIV14 primary cortical neurons transfected with scrambled, two Cyfip1 shRNAs (shRNA 319, 315), or shRNA 315 cotransfected with RNAi-resistant CYFIP1 WT, mutD, mutH, or mutE. Panels show representative dendritic sections; scale bar represents 5 µm. (Right Panel) The dendritic spine morphology of neurons shown on the left was categorized as mature (in black, stubby + mushroom-like) and immature (in white, long thin + filopodia). Shown is the percentage of mature and immature classes (at least ten neurons/condition, c2 test, ***$p < 0.001$). From De Rubeis et al., 2013.

All mutants failed to restore the normal spine distribution (Fig. 3), indicating that both CYFIP1 complexes are equally important for proper spine formation. In conclusion, CYFIP1 deficiency alters the proper functioning of two complexes modulating critical synaptic processes, i.e., protein synthesis and actin cytoskeleton remodeling, both of these ultimately leading to defects in spine morphology.

Aim 3: *Regulation of FMRP and CYFIP1 upon neuronal activity.*
Achievement 3: Translational initiation, in particular sequestration of eIF4E by 4E-binding proteins, is tightly controlled by the mTORC1 pathway. mTORC1 can be activated by a variety of receptors, including the metabotropic glutamate receptors (mGluRs). To investigate if this signaling cascade affects not only general 4E-BPs, but also specific ones such as CYFIP1, we stimulated acute hippocampal slices with a group I mGluRs agonist and simultaneously block mTORC1 with rapamycin. We found that CYFIP1 dissociates from eIF4E upon DHPG stimulation, but Rapamycin prevented this effect, thus indicating that mTORC1 is required to release CYFIP1 upon mGluRs. We are now currently studying the phosphorylation state of CYFIP1 by phosphoproteomics. We performed immunopurification of CYFIP1 from unstimulated and DHPG-treated synaptoneurosomes using a tandem affinity purification protocol (Totaro et al., 2011) that yields particularly pure immunocomplexes. The samples are currently being analysed in the laboratory of Prof. Kris Gevaert (University of Gent). These studies will allow us to understand how neuronal activity modulates the two complexes via posttranslational modifications.

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

Networking and collaborations

The project results from the integration of complementary expertise in our Institute and at other institutions abroad. First, the mass spectrometry analysis was conducted in collaboration with a group with a long lasting experience in mass spectrometry techniques, namely the group of Prof. August B. Smit and Dr. Ka Wan Li, at the VU University in Amsterdam, The Netherlands. Furthermore, to accomplish this work we have also collaborated with Dr. Danielle Posthuma, Department of Functional Genomics and Department of Medical Genomics, VU Amsterdam, The Netherlands who is a clinical geneticist and with European consortia and networks. For the FRET/FLIM measurement that revealed the conformational change in CYFIP1 (De Rubeis et al., 2013), we collaborated with the group of Dr. Daniel Choquet and Dr. Christel Poujol at the CNRS Interdisciplinary Institute for Neuroscience (IINS) and the University of Bordeaux, France. We also continued our long-lasting collaboration with Dr. Seth Grant at the University of Edinburgh, UK.

Finally, we are proficiently collaborating with the microscope imaging facility at the Center of Human Genetics, KU Leuven (Light Microscopy & Imaging Network, LiMoNe) for spine visualization and analysis. Last year we started a new collaboration with Prof. Kris Gevaert to further study the CYFIP1 regulation via the identification of post-translational modifications.

Relevance

The results we obtained over the last three years, supported by the FMRE-GSKE, greatly contributed to the current knowledge of CYFIP1 distribution and function/s in brain and provide novel mechanisms that explain the molecular aspects of several intellectual disabilities.

1. We have identified as partner of CYFIP1, new genes/proteins involved in Autism, Schizophrenia and other disabilities in humans.
2. We have increased our understanding on the function of synaptic compartment in the neuronal cells unraveling the different, interconnected CYFIP1 cellular functions.
3. We have shed light on the regulatory mechanisms tuning CYFIP1 complexes with neuronal stimulation.
4. We have provided evidence that the shaping and reshaping of the synapses occur through the coordinated function of CYFIP1 that orchestrates local protein synthesis and actin remodeling.

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

Publications supported by the F.M.R.E

- Daniele Di Marino, Tilmann Achsel, Caroline Lacoux, Mattia Falconi, and **Claudia Bagni** (2014). The severe Ile304Asn mutation in the Fragile X Mental Retardation Protein destabilizes the structure of the KH2 domain impairing its nucleic acid binding" ***J. Biomol. Struct. Dyn.,*** 32(3):337-50.
- Silvia De Rubeis, Emanuela Pasciuto, Ka Wan Li, Esperanza Fernández, Daniele Di Marino, Andrea Buzzi, Linnaea E. Ostroff, Eric Klann, Fried J.T. Zwartkruis, Noboru H. Komiyama, Seth G.N. Grant, Christel Poujol, Daniel Choquet, Tilmann Achsel, Danielle Posthuma, August B. Smit, and **Claudia Bagni** (2013). CYFIP1 Coordinates mRNA Translation and Cytoskeleton Remodeling to Ensure Proper Dendritic Spine Formation. ***Neuron*** 79(6):1169-82.
- Esperanza Fernández, Nicholas Rajan, and **Claudia Bagni** (2013).The FMRP regulon: from targets to disease convergence***. Front. Neurosci.*** 7:191.
- **Claudia Bagni,** Flora Tassone, Giovanni Neri and Randi Hagerman (2012). "Fragile X Syndrome Year 2012: Causes, Diagnosis, Mechanisms and Therapeutics". ***J. Clin. Invest.***,122: 4314-2.

Team publications (last five years).

- Daniele Di Marino, Tilmann Achsel, Caroline Lacoux, Mattia Falconi, and **Claudia Bagni** (2014). The severe Ile304Asn mutation in the Fragile X Mental Retardation Protein destabilizes the structure of the KH2 domain impairing its nucleic acid binding" ***J. Biomol. Struct. Dyn.,*** 32(3):337-50.
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- Silvia De Rubeis, Emanuela Pasciuto, Ka Wan Li, Esperanza Fernández, Daniele Di Marino, Andrea Buzzi, Linnaea E. Ostroff, Eric Klann, Fried J.T. Zwartkruis, Noboru H. Komiyama, Seth G.N. Grant, Christel Poujol, Daniel Choquet, Tilmann Achsel, Danielle Posthuma, August B. Smit, and **Claudia Bagni** (2013). CYFIP1 Coordinates mRNA Translation and Cytoskeleton Remodeling to Ensure Proper Dendritic Spine Formation. ***Neuron*** 79(6):1169-82
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Final report of the research group of

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

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

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

To gain a better understanding of Dmrt5 function, we further analysed its expression and examined the earliest consequences of its absence on cortical development. Our results show that Dmrt5 is already expressed from the neural plate stage in the prospective dorsal telencephalon. Using a ***BAT-gal*** reporter mice, we found that Wnt signaling is disrupted from stage E10.5 in the developing telencephalon of Dmrt5$^{-/-}$ embryos. By analyzing the transcriptome of the forebrain of Dmrt5$^{-/-}$ mice, we identified a few genes including the rostrolateral determinant ***Pax6*** gene that are already affected at E9.5 stage, suggesting that they may be regulated by Dmrt5 in a Wnt independent manner. Using a conditional Dmrt5 mutant, we found that Pax6 is rapidly upregulated in response to the loss of Dmrt5 and that it is required to repress Pax6 even at late stages of corticogenesis. Conversely, using ***in utero*** electroporation, we found that ***Dmrt5*** overexpression represses ***Pax6***. As ***Dmrt3*** is coexpressed with ***Dmrt5*** in the developing telencephalon, we also examined ***Pax6*** expression in ***Dmrt3***$^{-/-}$ mice and found that it is slightly upregulated compared to wild type mice. Together, our results suggest that ***Dmrt5*** promotes caudiomedial cortical development not only by promoting dorsal midline signaling but also by directly repressing some genes such as ***Pax6.*** They also provide the first evidence that ***Dmrt5*** and ***Dmrt3*** share a number of target genes in cortical progenitors. Chromatin immunoprecipitation experiments are currently underway to identify binding sites of Dmrt3-5 in cortical progenitors.

We also characterizated Dmrt5 in the frog ***Xenopus laevis***. As in the mouse, xDmrt5 is strongly expressed in the developing telencephalon and olfactory system. As the inductive events that lead to olfactory placode development remain unclear (Schlosser, 2006; Streit, 2008; Schlosser, 2010; Park et al., 2010), we analysed the regulatory inputs that control ***Dmrt5*** expression in the ectoderm. As Dmrt5 function in olfactory placode neurogenesis is unknown, we also analysed the consequences of its knockdown and overexpression on neurogenesis within the developing olfactory epithelium. Our results showed that ***Dmrt5*** gene is a novel important player in the developing olfactory system, induced by the events of neural induction and the integration of the inputs of the homeobox transcription factor Otx2 and of Notch signaling, and provide evidence for ***Dmrt5*** and ***Dmrt4*** redundant functions upstream of proneural factors. This work constitutes the first demonstration of the important role of Dmrt5 in neurogenesis during olfactory placode development. A manuscript by Parlier et al. entitled "**The Xenopus doublesex-related gene Dmrt5 is required for olfactory placode neurogenesis.** » describing those results has been published in ***Dev. Biol.***

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

Besides our work on cortical development, we have also studied neurogenesis in the spinal cord. Our work has been focused on two uncharacterized members of the Prdm family of transcription factors, ***Prdm12*** and ***Prdm13***, identified in an ***in situ*** hybridization screen in the frog embryo. This family of zinc finger transcription factors has recently spawned considerable interest as it has been implicated in fundamental aspects of cellular differentiation and exhibits expanding ties to human diseases (Fog et al., 2011 ; Hohenauer et al., 2012). We focused on those two genes because they are expressed in restricted progenitors of the developing CNS, which suggests a function for them in neuronal subtype specification.

Ptf1a is a basic helix-loop-helix (bHLH) proneural factor that plays a crucial role in the balance between GABAergic inhibitory neurons and glutamatergic excitatory neurons in different regions of the CNS, including the dorsal spinal cord. Our work identified ***Prdm13*** as a histone methyltransferase belonging to the ***Ptf1a*** synexpression group. Gain and loss of Ptf1a function analysis in both frog and mice indicates that ***Prdm13*** is positively regulated by Ptf1a and likely constitutes a direct transcriptional target. We also showed that this regulation requires the formation of the Ptf1a-Rbp-j complex. Prdm13 knockdown in ***Xenopus*** embryos and in ***Ptf1a*** overexpressing ectodermal explants lead to an upregulation of ***Tlx3/ Hox11L2,*** which specifies a glutamatergic lineage and a reduction of the GABAergic neuronal marker ***Pax2***. It also leads to an upregulation of ***Prdm13*** transcription, suggesting an autonegative regulation. Conversely, in animal caps, Prdm13 blocks the ability of the bHLH factor Neurog2, a target of Ptf1a, to activate ***Tlx3***. Additional gain of function experiments in the chick neural tube confirm that ***Prdm13*** suppresses $Tlx3^+$/glutamatergic and induces $Pax2^+$/GABAergic neuronal fate. Thus, Prdm13 is a novel crucial component of the Ptf1a regulatory pathway that, by modulating the transcriptional activity of bHLH factors such as Neurog2, controls the balance between GABAergic and glutamatergic neuronal fate in the dorsal and caudal part of the vertebrate neural tube. This work entitled "**The *Prdm13* histone methyltransferase encoding gene is a Ptf1a-Rbpj downstream target that suppresses glutamatergic and promotes GABAergic neuronal fate in the dorsal neural tube**" is currently in press in ***Dev. Biol.***

As for *Prdm12*, we have shown that *Prdm12* is expressed selectively in progenitors of V1 interneurons. To approach the mechanisms responsible for it selective expression in P1 progenitors, we have studied its expression in *Pax6* KO mice as *Pax6* is broadly expressed in the ventral neural tube. We also analyse its expression in *Dbx1* and *Nkx6.1* K0 mice because the limit of their domain of expression correspond to the p1 domain of progenitors. We found that *Prdm12* expression is lost in *Pax6* mutants and is expanded in *Dbx1* and *Nkx6.1* mutants, indicating that *Prdm12* is dependent on *Pax6* and is restricted to p1 due to the repressive action of Dbx1 dorsally and Nkx6.1 ventrally.

To approach its function, we perfom gain and loss of function experiments in the frog and chicken neural tube. The results obtained indicate that it plays a crucial role in the spinal cord in V1 interneuron (IN) specification. Its precise roles and mechanisms of action is currently under investigation.

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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
- extend our FTLD patient and matched control samples
- identify genes modifying onset age in FTLD
- identify novel causal genes for FTLD
- identify novel susceptibility genes for FTLD

The major achievements in this project were 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 amyotrophic lateral sclerosis (ALS). This novel mutation is exceptionally prevalent and explained up to 30% of patients. Moreover, the repeat expansion turned out to be an equally important cause of amyotrophic lateral sclerosis. We were major contributors to the genetic, clinical, neuropathological and biochemical characterization of this novel type of repeat expansion disorder. Extending on the strong overlap between genetics of FTLD and ALS, we also assessed the presence of FTLD mutations in some novel ALS genes. Further, we extended our knowledge on the biological function of granulin (*GRN*), the FTLD gene that we identified in 2006 [Cruts *et al.* 2006] in knock-out mice. Similarly, we fully characterized *TARDBP* overexpression mice that represent novel models for TDP-43 proteinopathies including FTLD and ALS.

We also contributed to the identification and genetic and clinical characterization of *TMEM106B*, the first common susceptibility gene found in FTLD. In addition, we have made significant progress in the research to identify onset age modifying genes in FTLD.

Finally, we reported on the Alzheimer disease and frontotemporal dementia (AD&FTLD) and Parkinson disease (PD) Mutation Databases that we maintain for many years. They are established resources for clinical geneticists, neurologists, and researchers in need of comprehensive, referenced genetic, epidemiologic, clinical, neuropathological, and/or cell biological information of specific gene mutations in these diseases. In addition, the aggregate analysis of all information available in the databases provides unique opportunities to extract mutation characteristics and genotype-phenotype correlations, which would be otherwise unnoticed and unexplored [Cruts *et al.* 2012; http://www.molgen.ua.ac.be/FTDMutations].

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]. We studied in detail the clinical and genetic characteristics of patients with *GRN*-associated FTLD. So far, all FTD-associated *GRN* mutations are leading to neurodegeneration through a "loss-of-function" mechanism, encouraging researchers to develop a growing number of cellular and animal models for GRN deficiency. GRN is a multifunctional secreted growth factor, and loss of its function can affect different cellular processes. Besides loss-of-function (i.e., mostly premature termination codons) mutations, which cause *GRN* haploinsufficiency through reduction of *GRN* expression, FTD-associated *GRN* missense mutations have also been identified. Several of these missense mutations are predicted to increase the risk of developing neurodegenerative diseases through altering various key biological properties of GRN-like protein secretion, proteolytic processing, and neurite outgrowth. With the use of cellular and animal models for GRN deficiency, the portfolio of GRN functions has recently been extended to include functions in important biological processes like energy and protein homeostasis, inflammation as well as neuronal survival, neurite outgrowth, and branching. Furthermore, GRN-deficient animal models have been established and they are believed to be promising disease models as they show accelerated aging and recapitulate at least some neuropathological features of FTD [Kleinberger *et al.* 2013]. 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.

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

Granulin (*GRN*)

Loss-of-function mutations in granulin (*GRN*) are associated with FTLD with intraneuronal ubiquitinated protein accumulations composed primarily of hyperphosphorylated TDP-43 (FTLD-TDP). 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].

We also analyzed whether DNA methylation in the *GRN* core promoter restricts *GRN* expression and, thus might promote FTLD in the absence of *GRN* mutations. *GRN* expression in human lymphoblast cell lines is negatively correlated with methylation at several CpG units within the *GRN* promoter. Chronic treatment with DNA methyltransferase inhibitor strongly induced mRNA and protein levels. In a reporter assay, CpG methylation blocked transcriptional activity of the *GRN* core promoter. In brains of FTLD patients several CpG units in the *GRN* promoter were significantly hypermethylated compared to age-matched healthy controls, Alzheimer and Parkinson patients. These CpG motifs were critical for *GRN* promoter activity in reporter assays. Furthermore, DNA methyltransferase 3a (DNMT3a) is upregulated in FTLD patients and overexpression of DNMT3a reduces GRN promoter activity and expression. Together, these data suggested that altered DNA methylation is a novel pathomechanism for FTLD that is potentially amenable to targeted pharmacotherapy [Banzhaf-Strathmann *et al.* 2013].

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

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. Following up on this locus, 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 (Figure 1). 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].

We assessed the geographical distribution of *C9orf72* G_4C_2 repeat expansions in a collection of 1,205 European FTLD patients, ascertained by the European Early-Onset Dementia (EOD) consortium. Next, we performed a meta-analysis of these data and that of other European studies, together 2,668 patients from 15 Western European countries. The frequency of *C9orf72* repeat expansions in Western Europe was 9.98%, with 18.52% in familial, and 6.26% in sporadic FTLD patients. Outliers were Finland and Sweden with overall frequencies of respectively 29.33% and 20.73%, but also Spain with 25.49%. In contrast, prevalence in Germany was limited to 4.82% [van der Zee, Gijselinck *et al.* 2013].

To characterize patients with frontotemporal lobar degeneration (FTLD) with a repeat expansion mutation in *C9orf72*, and to determine whether there are differences in the clinical presentation compared with FTLD carriers of a mutation in *GRN* or *MAPT* or with patients with FTLD without mutation, we studied 275genetically and phenotypically thoroughly characterized patients with FTLD. We compared clinical and demographic characteristics of 26 *C9orf72* expansion carriers with patients with a *GRN* or *MAPT* mutation, as well as patients with familial and sporadic FTLD without mutation. *C9orf72* expansion carriers developed FTLD at an early age (average, 55.3 years; range, 42-69 years), significantly earlier than in *GRN* mutation carriers or patients with FTLD without mutation. Mean survival (6.2 years; range, 1.5-17.0 years) was similar to other patient groups. Most developed behavioral variant frontotemporal dementia (85%), with disinhibited behavior as the prominent feature. Concomitant ALS is a strong distinguishing feature for *C9orf72*-associated FTLD. However, in most patients (73%), ALS symptoms were absent. Compared with *C9orf72* expansion carriers, nonfluent aphasia and limb apraxia were significantly more common in *GRN* mutation carriers. *C9orf72*-associated FTLD most often presents with early-onset behavioral variant frontotemporal dementia with disinhibition as the prominent feature, with or without ALS. Based on the observed genotype-phenotype correlations between the different FTLD syndromes and different genetic causes, we proposed a decision tree to guide clinical genetic testing in patients clinically diagnosed as having FTLD [Van Langenhove *et al.* 2013].

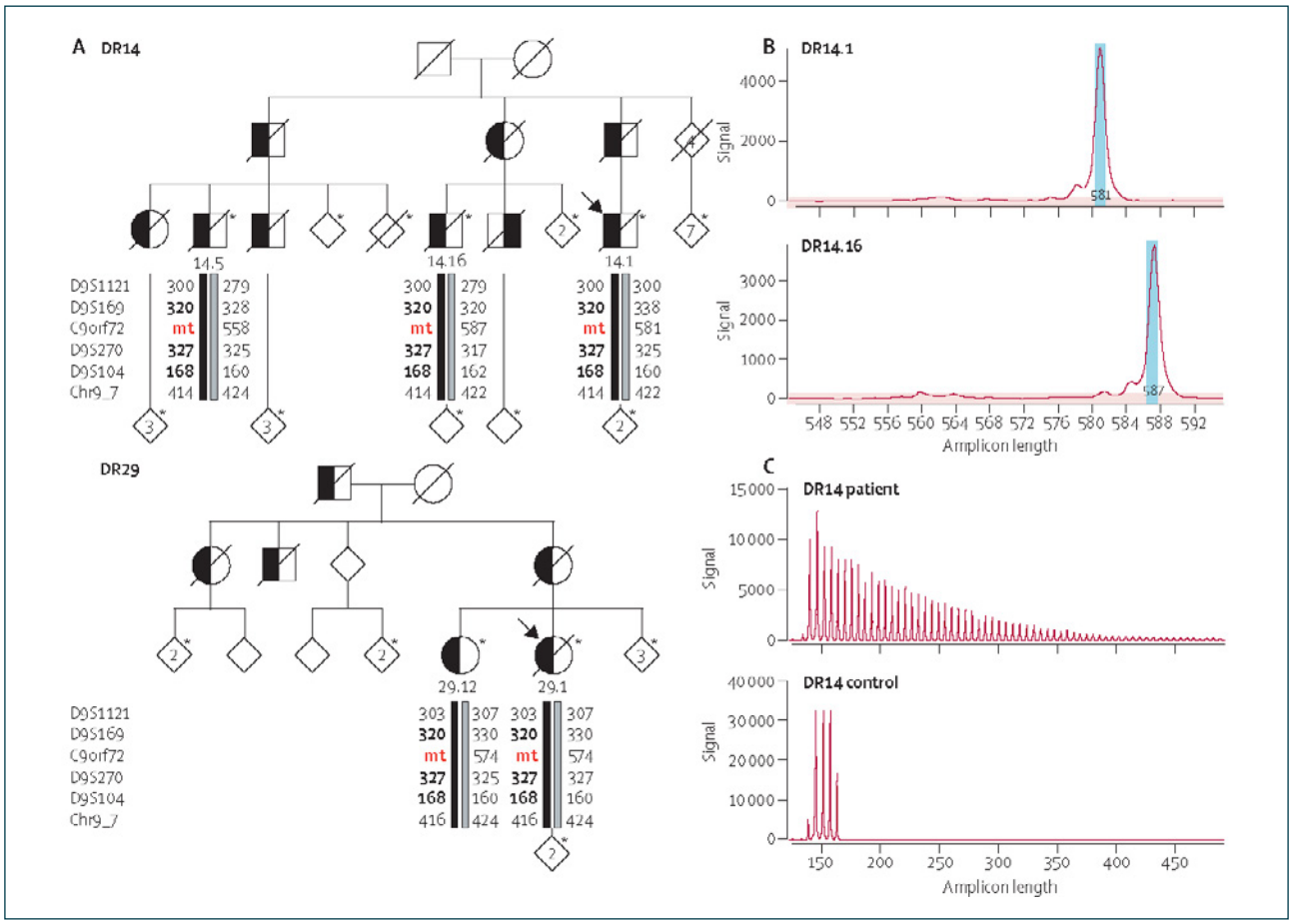

Figure 1 *C9orf72* repeat expansion in FTLD-ALS (**A**) Abbreviated pedigrees of genetically related families DR14 and DR29. Disease haplotypes segregating in the families are shown as black bars and the haplotype shared between families DR14 and DR29 are shown in bold. The *C9orf72* repeat is included 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].

Although primarily characterized by variably abundant pathological inclusions of TDP-43 protein, the lesion load was extended to TDP-43-negative, p62-positive neuronal and glial inclusions in extended regions of the central nervous system (CNS), particularly in cerebellum, where they may be characteristic of a *C9orf72* repeat expansion. Disease mechanisms associated with repeat expansion disorders, including haploinsufficiency, RNA toxicity, and abnormal translation of expanded repeat sequences (Figure 2), are beginning to emerge [Cruts *et al.* 2013].

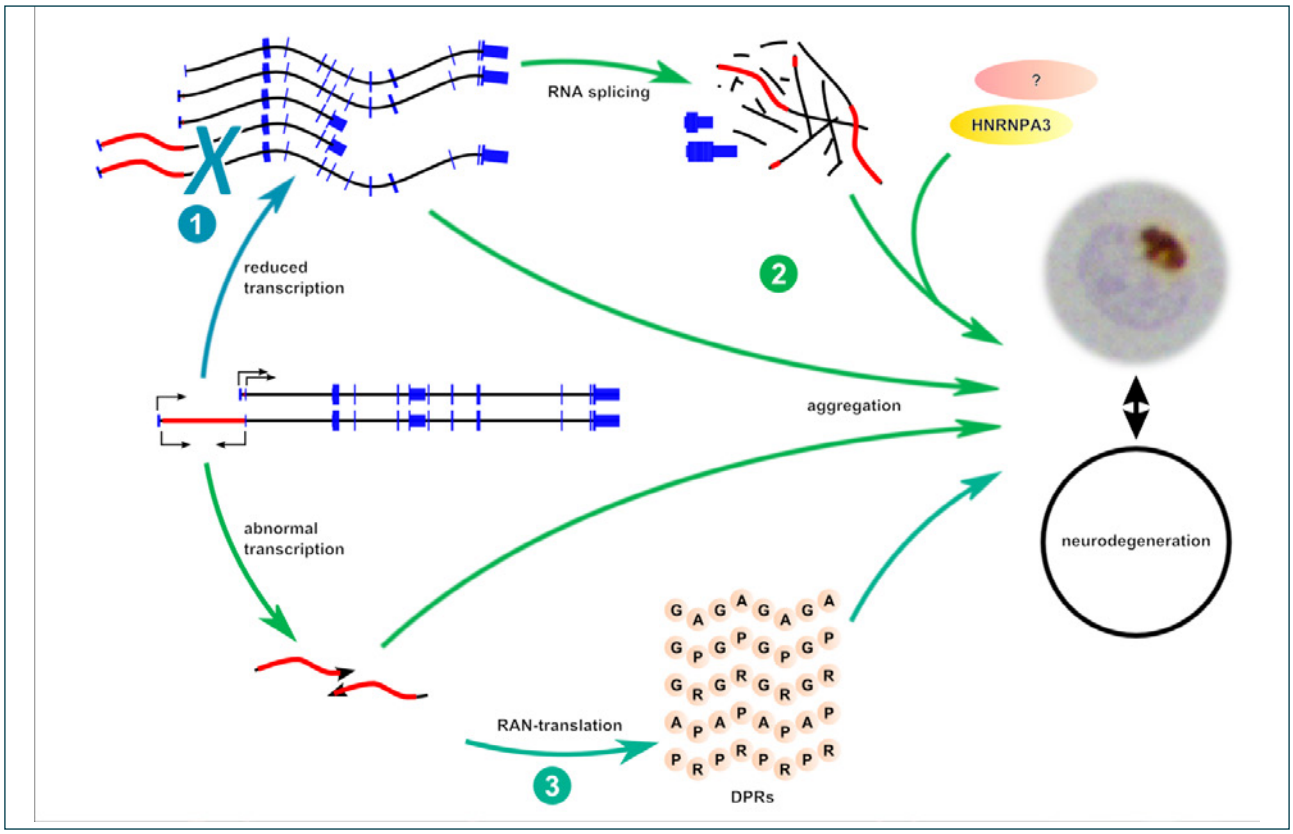

Figure 2 Putative, mutually nonexclusive pathological mechanisms associated with the hexanucleotide repeat expansion in C9orf72, for which some evidence was reported. (1) Haplo-insufficiency resulting from reduced expression of the gene copy located on the repeat-containing haplotype e.g. due to altered binding of transcription factors, DNA methylation and/or position effects. (2) RNA gain of function toxicity due to the formation of aggregates of repeat-containing RNA species and sequestered RNA-binding proteins. (3) abnormal bidirectional transcription of expanded repeat sequences followed by repeat-associated non-ATG-initiated (RAN) translation into aggregation-prone dipeptide repeat proteins (DPRs) [Cruts *et al*. 2013].

Intermediate repeat sizes (7 to 24 repeat units) are strongly correlated with a haplotype conferring significant risk especially for developing ALS and FTLD-TDP [van Es *et al.* 2009;Laaksovirta *et al.* 2010;Shatunov *et al.* 2010;Van Deerlin *et al.* 2010]. We studied the role of intermediate repeat sizes on *C9orf72* gene expression. *In vitro* reporter gene expression studies demonstrated significantly decreased transcriptional activity of *C9orf72* with increasing number of repeat units (Figure 3), indicating that intermediate repeats might act as predisposing alleles and in favor of the loss-of-function disease mechanism. Further, we observed a significantly increased frequency of short indels in the GC-rich low complexity sequence (LCS) adjacent to the G_4C_2 repeat in *C9orf72* expansion carriers ($p < 0.001$) with the most common indel creating one long contiguous imperfect G_4C_2 repeat which is likely more prone to replication slippage and pathological expansion [van der Zee, Gijselinck *et al.* 2013].

Since repeat expansions might cause RNA toxicity via sequestration of RNA-binding proteins, we searched for proteins capable of binding to G_4C_2 repeats. *In vitro* transcribed, biotinylated RNA containing hexanucleotide G_4C_2 or, as control, A_4C_2 repeats were incubated with nuclear protein

extracts. Using stringent filtering protocols, 20 RNA-binding proteins with a variety of different functions in RNA metabolism, translation and transport were identified. A subset of these proteins was further investigated by immunohistochemistry in human autopsy brains. This revealed that hnRNPA3 formed neuronal cytoplasmic and intranuclear inclusions in the hippocampus of patients with *C9orf72* repeat extensions. Confocal microcopy showed that these inclusions belong to the group of the so far enigmatic p62-positive, TDP-43 negative inclusions characteristically seen in autopsy cases of diseased *C9orf72* repeat expansion carriers. Thus, we have identified one protein component of these pathognomonic inclusions [Mori *et al.* 2013b].

As in other FTLD/ALS variants, characteristic intracellular inclusions of misfolded proteins define *C9orf72* pathology, but the core proteins of the majority of inclusions are still unknown. We found that most of these characteristic inclusions contain poly-(Gly-Ala) and, to a lesser extent, poly-(Gly-Pro) and poly-(Gly-Arg) dipeptide-repeat (DPR) proteins presumably generated by non-ATG-initiated translation from the expanded G_4C_2 repeat in three reading frames. These findings directly link the FTLD/ALS-associated genetic mutation to the predominant pathology in patients with C9orf72 hexanucleotide expansion [Mori *et al.* 2013c]. We took an antibody-based approach to further validate the translation of DPR proteins. To test whether the antisense repeat RNA transcript is also translated, we raised antibodies against the predicted products, poly-(Ala-Pro) and poly-(Pro-Arg). Both antibodies stained p62-positive neuronal cytoplasmic inclusions throughout the cerebellum and hippocampus indicating that not only sense but also antisense strand repeats are translated into DPR proteins in the absence of ATG start codons. Protein products of both strands co-aggregate suggesting concurrent translation of both strands. Moreover, an antibody targeting the putative carboxyl terminus of DPR proteins can detect inclusion pathology in *C9orf72* repeat expansion carriers suggesting that the non-ATG translation continues through the entire repeat and beyond. A highly sensitive monoclonal antibody against poly-(Gly-Arg), visualized abundant inclusion pathology in all cortical regions and some inclusions also in motoneurons. Together, our data show that the G_4C_2 repeat is bidirectionally translated into five distinct DPR proteins that co-aggregate in the characteristic p62-positive TDP-43 negative inclusions found in FTLD/ALS cases with *C9orf72* repeat expansion. Novel monoclonal antibodies against poly-(Gly-Arg) will facilitate pathological diagnosis of *C9orf72* FTLD/ALS [Mori *et al.* 2013a].

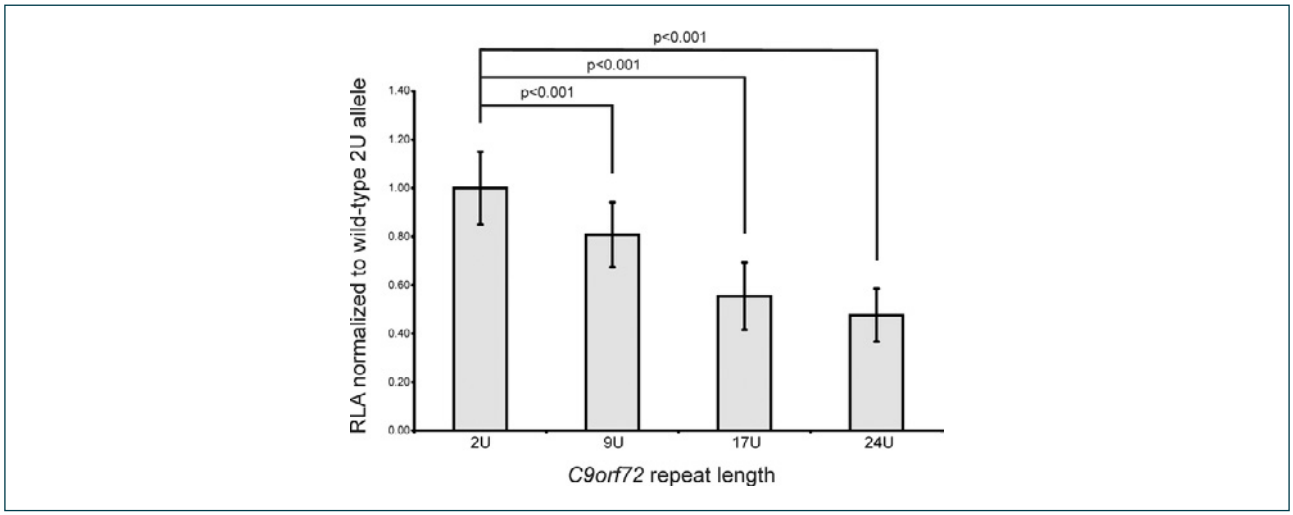

Figure 3. Transcriptional activity of C9orf72 promoter with alleles of different repeat length. Bars represent relative Gaussia/Cypridina luciferase activities (RLA) for the different C9orf72 constructs compared to the wild type allele of 2 units, for an increasing amount of repeat units. Values represent the mean (±SDEV) of 36 independent measurements relative to the 2 units wild type allele. The significance of differences in expression was calculated using the Mann-Whitney U test. P-values are presented above the bars [van der Zee, Gijselinck *et al.* 2013].

TARDBP

Aggregation of misfolded TDP-43 is a striking hallmark of neurodegenerative processes that are observed in several neurological disorders, and in particular in most FTLD or ALS patients. A direct causal link with TDP-43 brain proteinopathy was provided by the identification of pathogenic mutations in *TARDBP*, the gene encoding TDP-43, in ALS families. However, TDP-43 proteinopathy has also been observed in carriers of mutations in several other genes associated with both ALS and FTLD demonstrating a key role for TDP-43 in neurodegeneration. To date, and despite substantial research into the biology of TDP-43, its functioning in normal brain and in neurodegeneration processes remains largely elusive. Nonetheless, breakthroughs using cellular and animal models have provided valuable insights into ALS and FTLD pathogenesis. Accumulating evidence has redirected the research focus towards a major role for impaired RNA metabolism and protein homeostasis. At the same time, the concept that toxic TDP-43 protein aggregates promote neurodegeneration is losing its credibility. This review aims at highlighting and discussing the current knowledge on TDP-43 driven pathomechanisms leading to neurodegeneration as observed in TDP-43 proteinopathies. Based on the complexity of the associated neurological diseases, a clear understanding of the essential pathological modifications will be crucial for further therapeutic interventions [Janssens and Van Broeckhoven 2013]. To determine the physiological role of TDP43, we studied including zebrafish and mouse models.

We knocked out zebrafish tardbp and its paralogue tardbp-like, which lacks the glycine-rich domain where ALS and FTLD-TDP mutations cluster. tardbp mutants show no phenotype, a result of compensation by a unique splice variant of tardbpl that additionally contains a C-terminal elongation highly homologous to the glycine-rich domain of tardbp. Double-homozygous mutants of tardbp and tardbpl show muscle degeneration, strongly reduced blood circulation, mispatterning of vessels, impaired spinal motor neuron axon outgrowth, and early death. In double mutants the muscle-specific actin binding protein Filamin C a is up-regulated. Strikingly, Filamin C is similarly increased in the frontal cortex of FTLD-TDP patients, suggesting aberrant expression in smooth muscle cells and TDP-43 loss-of-function as one underlying disease mechanism [Schmid *et al.* 2013].

Various *in vitro* and *in vivo* studies have demonstrated toxicity of both mutant and wild-type TDP-43 to neuronal cells. To study the potential additional toxicity incurred by mutant TDP-43 *in vivo*, we generated mutant human TDP-43 (p.M337V) transgenic mouse lines driven by the Thy-1.2 promoter (Mt-TAR) and compared them in the same experimental setting to the disease phenotype observed in wild-type TDP-43 transgenic lines (wt-TAR) expressing comparable TDP-43 levels. Overexpression of mutant TDP-43 led to a worsened dose-dependent disease phenotype in terms of motor dysfunction, neurodegeneration, gliosis, and development of ubiquitin and phosphorylated TDP-43 pathology. Furthermore, we showed that cellular aggregate formation or accumulation of TDP-43 C-terminal fragments (CTFs) are not primarily responsible for development of the observed disease phenotype in both mutant and wild-type TDP-43 mice [Janssens *et al.* 2013].

UBQLN2 and *PFN1*

Because ubiquilin 2 (*UBQLN2*) and profilin 1 (*PFN1*) were recently associated ALS, we investigated a role for these genes in FTLD. We screened 328 FTLD, 17 FTLD-ALS, and 157 ALS patients. The frequency of *UBQLN2* and *PFN1* genetic variants in the FTLD patients was low at 0.30% and 0.91% respectively. Moreover, the biological relevance to disease of the variants was questionable. In *UBQLN2*,

we identified p.S346C outside of the PXX domain in 1 FTLD patient. Yet, a closely located serine substitution, p.S340I, was observed in a neurologically healthy control individual. In *PFN1*, we observed the previously reported p.E117G mutation in 3 FTLD patients and in 3 control individuals. In the ALS patient cohort, we detected *UBQLN2* variants in 1.27% of patients. These involved 2 novel missense mutations, p.S400G and p.P440L, that were also present in unaffected relatives. No mutations were observed in *PFN1*. We concluded that genetic variations in *UBQLN2* and *PFN1* in a predominantly Flanders-Belgian cohort of FTLD and ALS patients are extremely rare [Dillen *et al.* 2013].

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 Pvalue 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].

Other risk genes

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

Homozygous mutations in exon 2 of *TREM2*, a gene involved in Nasu-Hakola disease, can cause frontotemporal dementia (FTD). Moreover, a rare *TREM2* exon 2 variant (p.R47H) was reported to increase the risk of Alzheimer's disease (AD) with an odds ratio as strong as that for *APOEε4*. We systematically screened the *TREM2* coding region within a Belgian study on neurodegenerative brain diseases (1216 AD patients, 357 FTD patients, and 1094 controls). We observed an enrichment of rare variants across *TREM2* in both AD and FTD patients compared to controls, most notably in the extracellular IgV-set domain (relative risk = 3.84 [95% confidence interval = 1.29-11.44]; p = 0.009 for AD; relative risk = 6.19 [95% confidence interval = 1.86-20.61]; p = 0.0007 for FTD). None of the rare variants individually reached significant association, but the frequency of p.R47H was increased ~3-fold in both AD and FTD patients compared to controls, in line with previous reports. Meta-analysis including 11 previously screened AD cohorts confirmed the association of p.R47H with AD (p = 2.93×10(-17)). Our data corroborate and extend previous findings to include an increased frequency of rare heterozygous *TREM2* variations in AD and FTD, and show that *TREM2* variants may play a role in neurodegenerative diseases in general [Cuyvers *et al.* 2013].

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Articles in Books

2012

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Abstracts in Abstract books of International Meetings

2011

- 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)

2012

- **Gijselinck,I., van der Zee,J.,** Dillen,L., Van Langenhove,T., Philtjens,S., Engelborghs,S., Vandenbulcke,M., Bäumer,V., Maes,G., Santens,P., Cras,P., Robberecht,W., De Jonghe,P., Vandenberghe,R., De Deyn,P., Van Broeckhoven,C., **Cruts,M**.: Genomic Characterization of the C9orf72 Repeat Region Associated with FTLD and ALS. International Conference on Frontotemporal dementia, Manchester, UK, September 5-7 (2012) Dementia and Geriatric Cognitive Disorders 34(S1): 69
- **Gijselinck,I.**, Van Langenhove,T., **van der Zee,J.**, Philtjens,S., Engelborghs,S., De Jonghe,P., Vandenberghe,R., Santens,P., De Bleecker,J., Bäumer,V., Maes,G., Dillen,L., Cras,P., Robberecht,W., De Deyn,P., Van Broeckhoven,C., **Cruts,M.**: Functional effects of the MAPT haplotypes. Alzheimer's & Dementia - Supplement AAIC : (2012) (I.F.: 6.373)
- Philtjens,S., **Gijselinck,I.,** Van Langenhove,T., **van der Zee,J**., Engelborgs,S., Vandenberghe,R., Vandenbulcke,M., Santens,P., De Deyn,P., Van Broeckhoven,C**., Cruts,M**.: Identification of novel causal genes for frontotemporal lobar degeneration using whole genome sequencing. International Conference on Frontotemporal dementia, Manchester, UK, September 5-7 (2012) Dementia and Geriatric Cognitive Disorders 34(S1): 9
- Philtjens,S., **Gijselinck,I.**, Van Langenhove,T., **van der Zee,J.**, Engelborghs,S., Vandenberghe,R., Santens,P., De Deyn,P., Van Broeckhoven,C., **Cruts,M.**: In search of genetic defects in unrelated frontotemporal lobar degeneration patients using whole genome sequencing. European Journal of Human Genetics - Supplement ESHG P12.163: (2012) (I.F.: 4.4)
- **van der Zee,J., Gijselinck,I.,** Dillen,L., Van Langenhove,T., Sieben,A., Martin,J.J., **Cruts,M**., Van Broeckhoven,C., European EOD Consortium: A Pan-European study of the C9orf72 expansion associated with FTLD and ALS. International Conference on Frontotemporal dementia, Manchester, UK, September 5-7 (2012) Dementia and Geriatric Cognitive Disorders 34(S1): 78
- **van der Zee,J.**, **Gijselinck,I.**, Dillen,L., Van Langenhove,T., Sieben,A., Martin,J-J., **Cruts,M.**, Van Broeckhoven,C., EOD Consortium,: Geographical frequency of the FTLD-ALS causing C9Orf72 repeat expansion mutation in an extended cohort ascertained within the European Consortium on early-onset dementia. Alzheimer's & Dementia - Supplement AAIC : (2012) (I.F.: 6.373)

– Van Langenhove,T., **van der Zee,J., Gijselinck,I.**, Engelborghs,S., Vandenberghe,R., Vandenbulcke,M., De Bleecker,J., Sieben,A., Ivanoiu,A., Deryck,O., Willems,C., Martin,J-J., Michotte,A., Santens,P., De Jonghe,P., Cras,P., De Deyn,P., **Cruts,M.**, Van Broeckhoven,C.: Distinct clinical characteristics of C9orf72 expansion carriers compared to GRN, MAPT and non-mutation carries in a Flanders-Belgian FTLD cohort. International Conference on Frontotemporal dementia, Manchester, UK, September 5-7 (2012) Dementia and Geriatric Cognitive Disorders 34(S1): 226
– Van Langenhove,T., **van der Zee,J.**, **Gijselinck,I.**, Engelborghs,S., Vandenberghe,R., Sieben,A., Santens,P., De Jonghe,P., Cras,P., De Deyn,P., **Cruts,M.**, Van Broeckhoven,C., The Flanders Belgian Neurology network,: The clinical presentation of C9Orf72-associated frontotemporal lobar degeneration in an extended Flanders-Belgian cohort. Alzheimer's & Dementia - Supplement AAIC: (2012) (I.F.: 6.373)

2013

– Edbauer,D., Claus,R., Mücke,O., **van der Zee,J.**, Engelborghs,S., De Deyn,P.P., **Cruts,M.**, Van Broeckhoven,C., Plass,C., Strathmann,J.: Altered epigenetic regulation of progranulin (GRN) expression in frontotemporal lobar degeneration. 11th International Conference AD/PD, Florence, Italy, March 6-10, 2013 : (2013)
– Mori,K., Lammich,S., Mackenzie,I.R.A., Forné,I., Zilow,S., Kretzschmar,H., Edbauer,D., Janssens,J., Kleinberger,G., **Cruts,M.**, Herms,J, Neumann,M., Van Broeckhoven,C., Arzberger,T., Haass,C.: HNRNPA3 binds to GGGGCC repeats and is a constituent of pathognomic P62-positive inclusions in patients with C9ORF72 mutations. 11th International Conference AD/PD, Florence, Itatly, March 6-10, 2013 : (2013)
– Schmid,B., Hruscha,A., Strecker,K., Hogl,S., Strathmann,J., **van der Zee,J.**, Eimer,S., Kremmer,E., **Cruts,M.**, Edbauer,D., Van Broeckhoven,C., Lichtenthaler,S.F., Haass,C.: Loss of TDP-43 causes hypoperfusion, vessel mispatterning, and muscle degeneration. 11th International Conference AD/PD, Florence, Italy, March 6-10, 2013 : (2013)

Administrative Report

Honors, Prizes & Awards

Prizes

– **Cruts M.**: Medical Foundation Queen Elisabeth (GSKE), Solvay Prize, May 15, 2012
– **van der Zee J.:** AB Award of the King Baudouin Foundation, Brussels, Belgium, December 6, 2013

Travel Awards

International

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

National

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

Scientific Activities

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
– **Cruts M.:** organizer seminar SM Fendt, 'Understanding the cellular phenotype: the power of metabolomics and fluxomics' University of Antwerp, Belgium, May 7, 2013
– **Cruts M.:** organizer seminar D Prodanov, 'Electrical sensing and actuation interfaces for in vitro and In vivo applications', University of Antwerp, Belgium, April 30, 2013
– **Cruts M.:** organizer seminar. L Martens, 'An omics data ecosystem: proteomics data birth and (endless?) rebirth', University of Antwerp, Belgium, April 23, 2013

– **van der Zee J.:** EU-EOD consortium - 1st Annual Meeting of the European Early-Onset Dementia consortium, Antwerp, Belgium, June 12-14, 2013, Vice coordinator

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
– **Cruts M.**: 'Weeding Mendel's Garden: Can We Hoe Dubious Genetic Associations?', Alzforum Webinar Panel Discussion, July 31, 2012
– **van der Zee J.:** Invited Session Speaker 'Genetisch onderzoek naar Dementie brengt nieuwe inzichten', Research Foundation Flanders (FWO) Kennismakers, 85 jaar zuurstof voor Onderzoek en Ontwikkeling, Ghent, Belgium, December 17, 2013

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
– **van der Zee J.:** "Recent advances in Genetics of dementia", Interuniversity neurology course, Université Libre de Bruxelles - Hôpital Erasme, Brussels, Belgium, June 16, 2012
– **van der Zee J.:** 'Translational genetics approaches into early-onset dementia', Kick-Off Scientific IAP P7/16 Meeting, Antwerpen, May 6, 2013
– **van der Zee J.:** Stichting Alzheimer onderzoek (SAO-FRA) en Petercam: 'Wat is dementie en hoever staat het wetenschappelijk onderzoek? ', Gent, May 7, 2013
– **van der Zee J.:** Infodag over Frontotemporale Dementie: 'Genetica van Frontotemporale Dementie', Antwerpen, Belgium, September 7, 2013

Oral Presentations – Slide Sessions

International

– **van der Zee J.:** "A European consortium for high-profile translational research on early-onset dementia – a prevalence study of the FTLD-ALS causing C9orf72 repeat expansion mutation in an extended European cohort", American Academy of Neurology 64th Annual Meeting 2012, New Orleans, LA, USA, April 21-28, 2012
– **van der Zee J.:** "Geographical frequency of the FTLD-ALS causing C9Orf72 repeat expansion mutation in an extended cohort ascertained within the European Consortium on early-onset dementia", Alzheimer's Association International Conference 2012, Vancouver, British Columbia, July 14-19, 2012

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

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
– **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
– **Gijselinck I.:** "Genomic characterization of the *C9Orf72* promoter repeat in FTLD and ALS patients", Alzheimer's Association International Conference 212, Vancouver, British-Columbia, July 14-19, 2012
– **Gijselinck I.:** "Genomic characterization of the *C9orf72* repeat region associated with FTLD and ALS", 8th International Conference on Frontotemporal Dementias, Manchester, UK, September 5-7, 2012
– **van der Zee J.:** "A Pan-European study of the *C9orf72* expansion associated with FTLD and ALS", 8th International Conference on Frontotemporal Dementias, Manchester, UK, September 5-7, 2012

– **van der Zee J.:** "A Pan-European study of the pathological C9orf72 hexanucleotide (G_4C_2) expansion associated with frontotemporal lobar degeneration and amyotrophic lateral sclerosis", American Society of Human Genetics (ASHG) Annual Meeting, San Francisco, November 6-10, 2012

National

– **Gijselinck I.:** "Genomic characterization of the *C9orf72* promoter repeat in FTLD and ALS patients", VIB Seminar 2012, Blankenberge, Belgium, April 18-20, 2012

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 der Zee J.:** Lay public presentation: "Dementie en het genetisch onderzoek", Davidsfonds, Ieper, Belgium, March 27, 2012
– **van der Zee J.:** Lay public presentation: "De Ziekte van Alzheimer, nieuwe wetenschappelijke inzichten rond dementie", Beurs 55+, Deurne, Belgium, May 30, 2012
– **van der Zee J.:** Lay public presentation: "Wetenschappelijk onderzoek naar Dementie", Openbare ouderenraad, Deurne, Belgium, October 15, 2012
– **van der Zee J.:** Dienstencentrum Portugesehof: 'Dementie en het genetisch onderzoek', Hoboken, February 4, 2013
– **van der Zee J.:** Regioscholing specialist ouderengeneeskunde regio Zuidwest-Brabant en Zeeland: 'Het genetisch onderzoek naar dementie', Kamperduiden, Nederland, November 4, 2013
– **van der Zee J.:** Professional Women's Association, Kasteel van Brasschaat: 'De Ziekte van Alzheimer, nieuwe wetenschappelijke inzichten rond dementie', Brasschaat, November 27, 2013

Final report of the research group of

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Applicant

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

Mowat-Wilson syndrome, a severe neurodevelopmental human disorder, and its emerging connection with pitt-hopkins syndrome

It is striking that several neurodevelopmental disorders that are caused by mutations in distinct genes display remarkably similar phenotypes, suggesting that the defective genes impinge at the cellular level on common processes. Mowat-Wilson syndrome (MWS, MIM #235730) and Pitt-Hopkins syndrome (PTHS, MIM #610954) are such disorders.

The significant support from the QEMF in the period 2011-2013 has enabled us to provide solid ground for taking our present work towards elucidating the common and unique features of MWS and, more recently, PTHS. For this, we continue to use mouse models, but are expanding this work to disease modeling in cultures of embryonic stem cells (ESCs) and neural stem cells (NSCs; with R. Poot, Rotterdam). It is further combined with biochemical studies in both our Leuven and more recently our Rotterdam-based labs. These studies involve

1. structure-function studies of Sip1 in order to define the role of Sip1 interaction with partner proteins, including Smad proteins and chromatin remodelers,
2. the mapping of the protein network/s in which the transcription factors (TFs) Sip1 and Tcf4 (*see below*) act,
3. the identification of their genome-wide target genes,
4. in their dynamic chromatin landscape.

In addition, these studies of MWS in mouse models have meanwhile enabled us to move also beyond those in neurodevelopment, *e.g.* in hematopoiesis and cancer.

Mutations in one of the two alleles of *ZFHX1B* (chr2q22, 120kb, 10 exons), encoding a 1,214aa-long protein in humans [named also SIP1 and ZEB2] cause **Mowat-Wilson syndrome** (MWS; www.mowatwilson.org) [1-6]. One of the most characteristic clinical signs in MWS is a distinctive deviant facial appearance along with severe intellectual disability (ID). The precise incidence of MWS, suggested to be 1/4,500 live births, is unknown, but thought to be under recognized [7]. This single-gene disorder is characterized by various malformations that do not appear all in all patients. They occur clearly in the central nervous system (CNS) [causing ID, delayed motor development, absence of corpus callosum, microcephaly, occurrence of seizures and epilepsy] and combine with developmental defects in the neural crest cell lineage [leading to cranio-facial abnormalities, Hirschsprung disease (HD)] and a wide and heterogeneous spectrum of other congenital anomalies such as hypotonia and eye and heart defects. Analysis of about 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.

During the course of our QEMF project, and in the context of a movie made by our laboratory for the celebration of the Prize Janine and Jacques Delruelle, awarded in 2013 by the QEMF to our work, we established contact and further dialogue with the families of MWS patients, mainly via www.mowatwilson.org, and with the clinicians. Indeed, some of our observations in our mouse models, in particular on the role of Sip1 in new developmental processes, make the clinicians to reinvestigate their patients.

Pitt-Hopkins syndrome was first described in 1978 [8]. Since 2007, about 110 PTHS patients have been diagnosed with heterozygous mutations or deletions of *TCF4* [for an overview, see [9]; see also the database at www.LOVD.nl/TCF4]. PTHS is, like MWS, characterized by severe ID and typical facial gestalt and many patients have seizures/epilepsy. Like in MWS, patients appear happy and smiling but have significant delays in developing speech and acquiring motor milestones. Clinical diagnosis of PTHS is still difficult, especially when facial gestalt is less typical, and the clinical presentation also resembles Rett (RTT; MIM #312750), Angelman (AS; MIM #105830) or, indeed, Mowat-Wilson syndromes. The gene encoding Transcription Factor 4 (TCF4, on chr18q21; also named bHLHb19, E2-2, ITF2, SEF2-1B] was identified as the disease-causing gene in patients with *de novo* mutations in this gene [10-12]. Most of the mutations are truncating or occur in the gene exons coding for the helix-loop-helix (HLH) moiety of this protein of the basic HLH family of TFs, suggesting that interaction of TCF4 with other proteins is important for its function [13]. WE have recently obtained evidence that SIP1 and TCF4 co-operate (Poot and Huylebroeck, unpublished).

In addition to the similarities between MWS and PTHS in patients, the **knockout (KO) mice** for *Sip1* and *Tcf4* are both embryonic lethal [14, 15]. Both TFs bind to E-box containing regulatory regions of candidate target genes and have multiple interaction partners.

Some of our previous work

Our lab was the first to discover the protein Sip1 in a two-hybrid screen for Smad-interacting proteins (SIPs) in yeast by virtue of its binding to the MH2 domain of the BMP effector protein Smad1 [16]. Subsequent work has shown that Sip1 binds to the TGFβ/nodal/activin Smads2/3 and the BMP-Smads1/5/8 in ligand-stimulated cells only, although many of Sip1's functions may be Smad-independent and hence underpin multiple mechanisms of action of Sip1. It is a DNA-binding TF that represses target gene transcription through binding with each of its two zinc finger clusters to a separated repeat of CACCT(G) (similar to E-boxes) in regulatory regions of genes [17]. Full-length Sip1 binds to the co-repressor CtBP [18] and the chromatin-remodeling co-repressor complex NuRD [19]. It can become an activator of transcription as well by binding to P300/PCAF [20].

Removal of both *Sip1* alleles from the entire mouse embryo results in embryonic lethality at E9.5, severe malformation of the neural plate, defective neural crest migration and defects in somitogenesis [14, 21, 22]. Following these studies, we have systematically knocked out Sip1 in different developing organs, demonstrating its major function in cranio-facial development [22], the enteric nervous system [23], myelination [24] and embryonic hematopoiesis [25].

Of particular interest to this QEMF supported project, we also found that **Sip1 is a major player in brain development**. Studies in collaboration with V. Tarabykin (Göttingen and Berlin) showed that Sip1 is involved in early development of the hippocampus [26], and that, in the cortical plate, it controls the expression level of secreted factors that feed back to cortical progenitors instructing them to generate neurons and astrocytes in a timely fashion [27]. Our most recent studies, performed with support of the QEMF, made a connection between the seizures and epilepsy characteristic to MWS and a deficit in migration of cortical interneurons in Sip1 conditional (c) KO mice [28]. We found that Sip1 controls in these cells the mRNA expression level of the guidance receptor Unc5b, which provides a directional cue to the newborn cortical interneurons [28, 29]. Furthermore, Sip1 drives the expression of several cortical interneuron-specific genes and seems to be a critical factor in the establishment of cortical interneuron

(sub)type identity [28, 29]. In addition, these studies of MWS in mouse models have meanwhile enabled us to move also beyond neurodevelopment, *e.g.* in adult hematopoiesis and cancer (with V. Janzen, Bonn; J. Haigh, Melbourne; S. Goossens and G. Berx, UGent; J. Meijerink, Rotterdam; J. Soulier, Paris; A. Goldrath, La Jolla) [Denecker *et al.*, *in revision*; Goossens *et al.*, *in revision*].

Work performed with the support of the QEMF

Our work performed in the context of the QEMF funding mainly used a combination of studies with various *Sip1* cKO and tissue/organ explants thereof, and cell culture. The resulting publications are listed below in a separate section of this final report.

The major focus in the **first phase of the QEMF project** 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 *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.*, 2012]. In addition, in collaborative work with F. Clotman (UC Louvain, Brussels) we have also found a new role for Sip1 in a subgroup of motor neurons in the spinal cord [Roy *et al.*, 2012].

In the **second phase of the QEMF project**, we then successfully finished our studies of Sip1 in the embryonic ventral forebrain, where we investigated the molecular mechanisms underlying Sip1's essential functions that regulate the tangential migration of GABAergic interneurons, which relates to seizures and epilepsy seen in MWS patients [van den Berghe *et al.*, 2013; McKinsey *et al.,* 2013].

Other QEMF supported work was in the area of human genetics, where our team did essential experiments on gene expression analysis in the brain cortex in the context of Nicolaides-Baraitser syndrome, which displays CNS defects [Van Houdt *et al.*, 2012]. Last but not least, another SIP, the novel protein Tdp2, turned out to be a unique DNA repair protein [Gomez-Herreros *et al.*, 2013], which we now study in neurodevelopment and in neurodegeneration, using our *Tdp2* KO mice.

1. Sip1 controls pain sensitivity, and reduced Sip1 levels cause hypoalgesia

In collaboration with the German Mouse Clinic (www.mouseclinic.de) in Munich, where we shipped a large cohort of control and adult *Sip1* heterozygous (+/-), carrying a mutant *Sip1* allele that is conceptually comparable to those in MWS patients, for phenotyping. In some of their assays, they 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 in our Leuven lab both floxed *Sip1* alleles from neural crest cell (NCC) precursors by using a Wnt1-Cre *"deleter"* strain. Doing so, we discovered 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 acknowledged, 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 KO mouse embryos.

These embryological data 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 this work indicates that Sip1 regulates thermal pain sensitivity [Pradier *et al.*, 2014] by controlling the transduction properties of nociceptive primary sensory neurons in a novel manner, *i.e.* 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. This prompted them to engage in studies aiming at documenting the possible role of Sip1 as BMP-Smad activity modulator in myelination. The basic HLH TFs Olig1 and Olig2 are known to promote myelination, whereas BMP and Wnt/β-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*-/- 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, we decided to generate with the team of R. Lu (Dallas) OG-lineage specific *Sip1* KO mice (using Olig1-Cre mice developed by the Lu team). Such *Sip1* KO mice develop generalized tremors, hind limb 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/β-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* gene promoter) as a candidate direct target gene for Sip1.

Further work documented that (Sip1-induced) Smad7 is required for OG differentiation and, downstream of Sip1, and like Sip1, promotes myelination by blocking the BMP and Wnt/β-catenin 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/β-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 β-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/β-catenin signaling, while β-catenin stabilization inhibits OG myelination.

So, our study [Weng *et al.*, 2012] 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 cKO mouse model for studies of Sip1 in different embryonic regions, including the spinal cord. In parallel, colleague F. Clotman (UC Louvain, 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.*, 2012].

During development, spinal motor neurons (MN) diversify into a variety of subtypes specifically dedicated to the control of particular sets of skeletal muscles or visceral organs. MN diversification depends on the coordinated action of 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 MNs 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;Sip1 mutant 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 and their subtype fate 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 [27]. We have extended studies on Sip1 in a second 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. We realized that a lot of questions on the precise actors and their regulation in migration and guidance of these cortical INs remain unanswered, which prompted us to enter such studies for the first time in our lab.

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 revealed that Sip1 (in a cell-autonomous fashion) is crucial for interpretation of Netrin family guidance cues (the majority of which are up regulated in the absence of Sip1) and not for their migratory capacity *per se*. The work also revealed a relation to seizures and epilepsy observed in the majority of MWS patients.

The major urgent objective was to validate the aberrant modulation in Sip1-deficient forebrain of a number of Netrin family proteins and their receptors, as suggested by the RNA-Seq. For this, our team has set up focal electroporation (FEP) in organotypic brain slices made from E13.5 wild-type, *Sip1*$^{flex7/flex7}$ and *Sip1* KO 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 these 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, we could conclude that Sip1 regulates important, selected guidance cues of the Netrin/Netrin receptor family [van den Berghe *et al.*, 2013; McKinsey *et al.*, 2013].

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, but also for embryonic haematopoiesis and myelination. 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 [27]. These

findings in studies with Sip1 were therefore also discussed in a published manuscript, in a broader context of TGFβ/BMP family Smad signalling [Conidi, Cazzola *et al.*, 2011].

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Invited lectures where research supported by the QEMF was/will be presented and the support was/will be acknowledged

DH: Danny Huylebroeck / ES: Eve Seuntjens

Sep.16-20, 2014:	DH: 10th International BMP Conference, Berlin (orgs P Knaus, D Graf, T. Müller), Berlin-Dahlem, Germany (invited speaker)
2014:	DH: Bridge Meetings 2013-2014 (org: F van Vliet), ErasmusMC, Rotterdam (invited speaker)
Oct. 18-19, 2013:	DH: Autumn meeting of the Belgian Society for Cell and Developmental Biology on "Experimental models of human diseases", Liège (org: A Chariot & L. Nguyen) (invited speaker)
Jan.30-Feb1,2013:	DH: SyBoss Meeting 2013: Systems Biology of Stem Cells, Kirchberg, Austria (orgs: F Stewart, A Beyer) (invited speaker)
Nov. 7, 2013:	DH: PostDoc Network Meeting ErasmusMC, Rotterdam (invited speaker)
Nov. 4, 2013:	DH: Center for Medical Genetics, UGent, Gent (P Coucke / A De Paepe)
Aug. 9, 2013:	DH: Summer School Master of Neuroscience, ErasmusMC (org: J van der Steen), Rotterdam, Aug. 5-23, 2013 (invited speaker)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)Dec. 6, 2012: Max-Planck Institute for Molecular Biomedicine, Dept for Cell and Developmental Biology, Münster, Germany (H Schöler)
Nov. 23, 2012:	DH: Swiss Center for Regenerative Medicine, Institute for Oral Biology, University Zurich, Switzerland (D Graf, T Mitsiadis)
Apr. 17, 2012:	DH: Seminar at Dept Biological Sciences and Biotechnology, Tsinghua University, Beijing, China (Y-G Chen)
Mar. 9, 2012:	DH: Seminar at the Institute for Chemistry and Biochemistry, Freie Universität Berlin, Germany (P Knaus)
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 TGFβ (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 TGFβ 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)

Final 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

Introduction

During these 3 years, our research followed 4 related streams:

- The characterization of spontaneous brain activity during wakefulness, sleep onset, sleep and anaesthesia
- The functional relationships between sleep and memory processing
- The regulation of sleep/wakefulness rhythms
- The non-visual influence of light upon cognitive brain responses.

These 4 research streams are detailed in the following sections.

Characterization of spontaneous brain activity during normal human sleep and anesthesia

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.

Schrouff J, Perlbarg V, Boly M, Marrelec G, Boveroux P, Vanhaudenhuyse A, Bruno MA, Laureys S, Phillips C, Pelegrini-Issac M, Maquet P, Benali H (2011) Brain functional integration decreases during propofol-induced loss of consciousness. Neuroimage 57:198-205.

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 (see Figure 1).

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

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 (see Figure 2).

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.

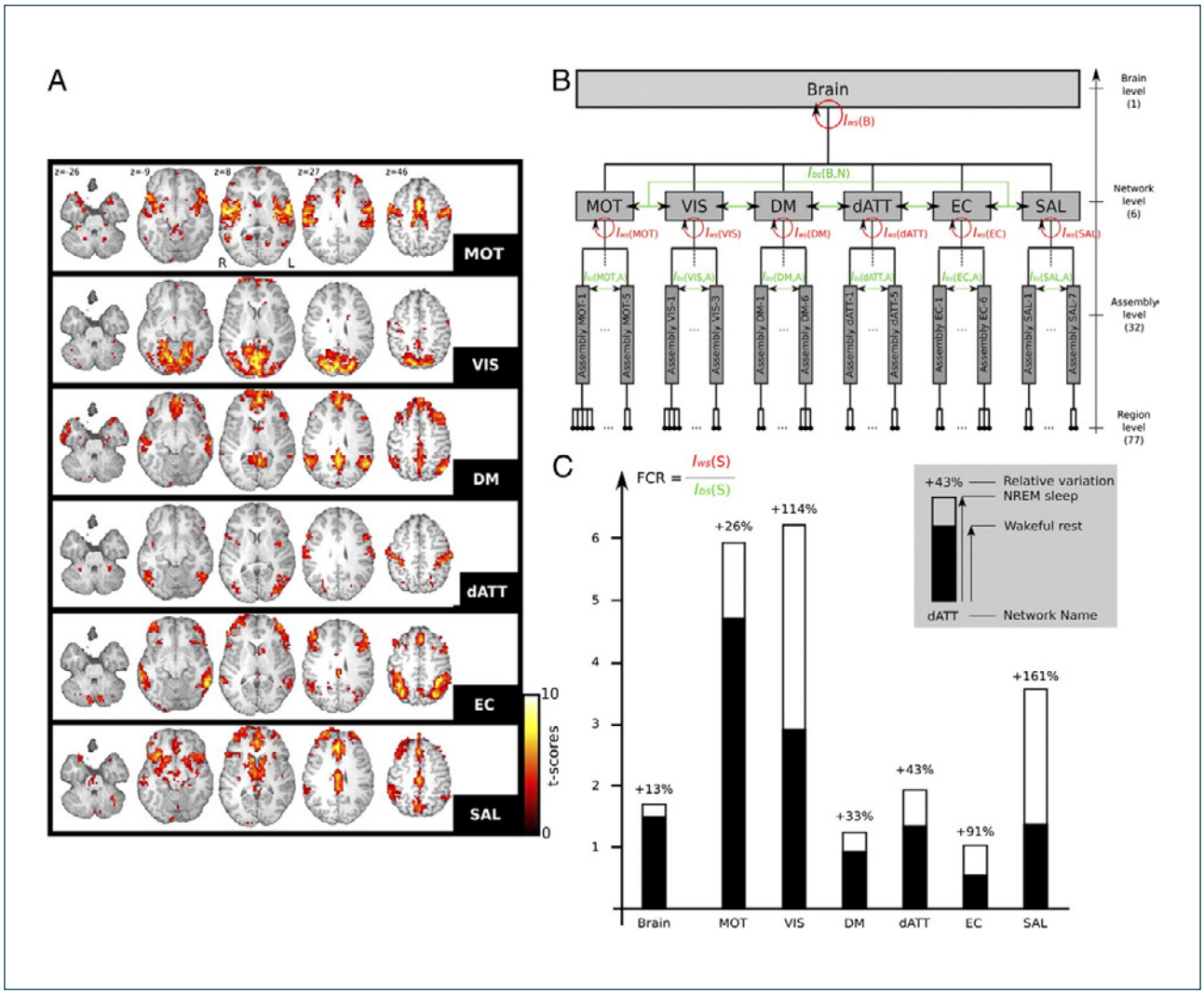

Figure 1. Summary of the results. (A) Six functional networks were extracted from data acquired during wakefulness, using independent component analysis. (B) Our method estimates integration in a hierarchical way, the brain being constituted of several networks that, in turn, are composed of assemblies of brain areas. (C) Functional clustering ratio is increased in the brain and the six networks during sleep as compared to wakefulness (all significant with a probability >0.95). Networks: dATT, dorsal attentional; DM, default mode; EC, executive control; MOT, sensorimotor; SAL, salience; VIS, visual.

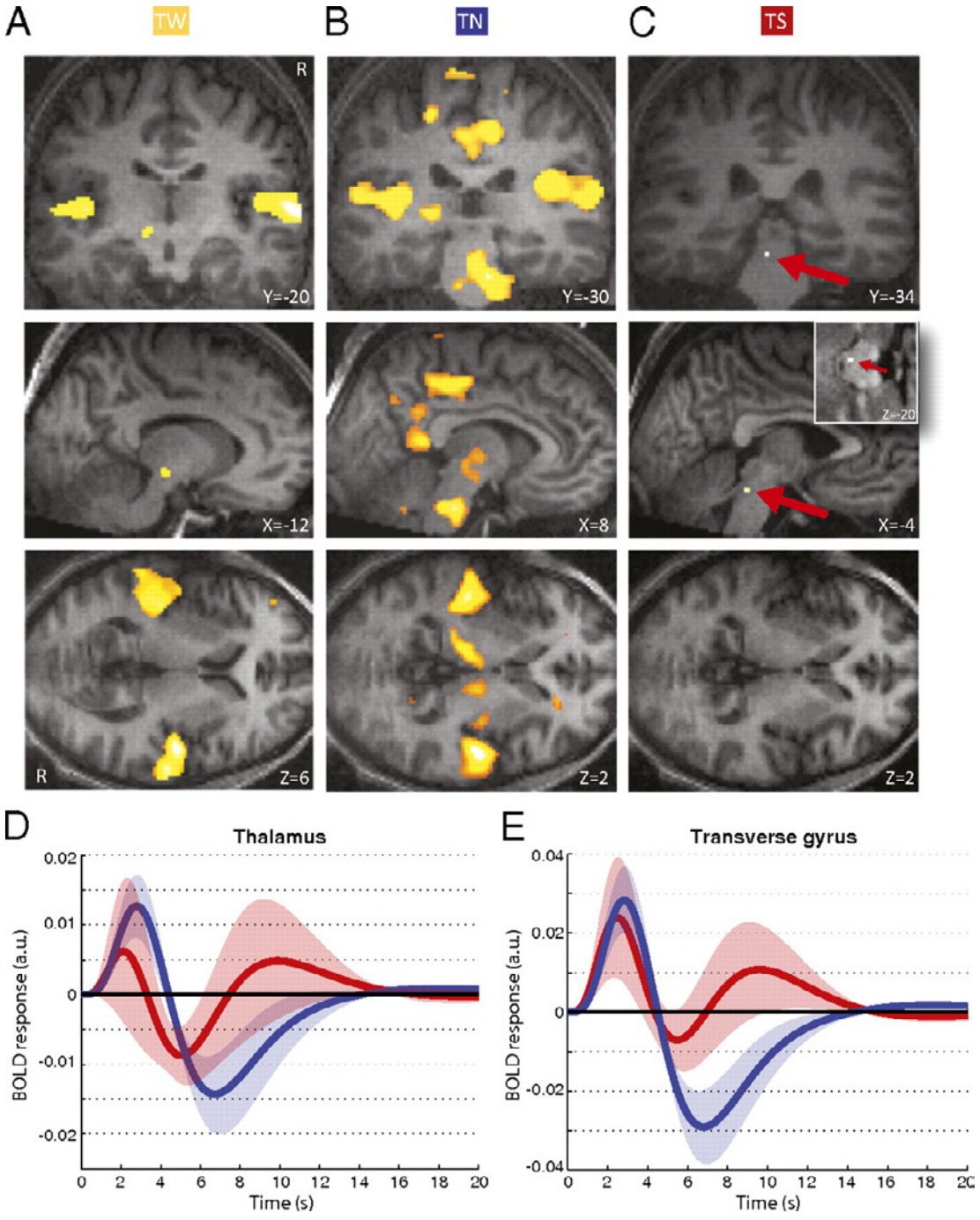

Figure 2. Brain regions activated in relation to tones during waking (TW), NREM sleep (outside spindles; TN), and spindles (TS). (A) Significant responses associated with tones presented during waking. (B) Significant responses associated with tones presented during S2-3 NREM sleep, in the absence of ongoing spindles. These responses are located in the thalamus, primary auditory cortex, brainstem, cerebellum, middle frontal gyrus, precuneus, posterior cingulate gyrus and the brainstem. (C) Significant responses associated with tones presented within spindles in S2-3 NREM sleep. In this area of the brainstem (arrow, Inset), neural populations that process sound are found in the nuclei of the lateral lemniscus. (D and E) Fitted responses in the thalamus (x = –12, y = –22, z = –6; D) and the auditory cortex (x = 58, y = –14, z = 6; E) associated with sounds delivered with (red, TS) or without (blue, TN) ongoing spontaneous spindle. The curves correspond to the mean and the shaded areas to the SEM. Coordinates of the thalamus and auditory cortex were derived from the peak voxels in these areas during wakefulness (TW).

We also identified the neurophysiological responses associated with auditory stimulation during different phases of slow waves of non-rapid eye movement (NREM) sleep using simultaneous electroencephalography (EEG)/functional magnetic resonance imaging (fMRI) recordings. It was reported earlier that auditory stimuli produce bilateral activation in auditory cortex, thalamus, and

caudate during both wakefulness and NREM sleep. However, due to the spontaneous membrane potential fluctuations cortical responses may be highly variable during NREM. Here we now examine the modulation of cerebral responses to tones depending on the phase of the slow oscillation. Thirteen healthy young subjects were scanned successfully during stage 2-4 NREM sleep in the first half of the night in a 3 T scanner. Subjects were not sleep-deprived and sounds were post hoc classified according to (i) the presence of sleep spindles or (ii) the phase of the slow oscillation during (+/-300 ms) tone delivery. These detected sounds were then entered as regressors of interest in fMRI analyses. Interestingly wake-like responses - although somewhat altered in size and location - persisted during NREM sleep, except during present spindles (as previously published in Dang-Vu et al., 2011) and the negative going phase of the slow oscillation during which responses became less consistent or even absent. While the phase of the slow oscillation did not alter brain responses in primary sensory cortex, it did modulate responses at higher cortical levels. In addition EEG analyses show a distinct N550 response to tones during the presence of light sleep spindles and suggest that in deep NREM sleep the brain is more responsive during the positive going slope of the slow oscillation. The presence of short temporal windows during which the brain is open to external stimuli is consistent with the fact that even during deep sleep meaningful events can be detected. Altogether, our results emphasize the notion that spontaneous fluctuations of brain activity profoundly modify brain responses to external information across all behavioral states, including deep NREM sleep.

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

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 21(1): 10-20.

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.

The functional relationships between sleep and memory processing

Sleep is believed to participate in memory processing by 2 distinct processes: a synaptic re-calibration through the generation of slow waves and the replay of learned neural activity pattern between hippocampal and neocortical areas. We tested these hypotheses is several fMRI studies.

During non-rapid eye movement (NREM) sleep, a global decrease in synaptic strength associated with slow waves (SWs) would enhance signal-to-noise ratio of neural responses during subsequent wakefulness. To test this prediction, 32 human volunteers were trained to a coarse orientation discrimination task, in either the morning or evening. They were retested after 8 h of wakefulness or sleep, respectively. Performance was enhanced only after a night of sleep, in the absence of any change in the abundance of NREM SWs but in proportion to the number of SWs "initiated" in lateral occipital areas during post-training NREM sleep. The sources of these waves overlapped with the lateral occipital complex, in which responses to the learned stimulus, as assessed by fMRI, were selectively increased the next morning. This response enhancement was proportional to rapid eye movement (REM) sleep duration. These results provide an example of local sleep in which local initiation of SWs during NREM sleep predicts later skill improvement and foreshadows locally enhanced neural signals the next day. In addition, REM sleep also promotes local learning-dependent activity, possibly by promoting synaptic plasticity (see Figure 3).

Mascetti, L., Muto, V., Matarazzo, L., Foret, A., Ziegler, E., Albouy, G., Sterpenich, V., Schmidt, C., Degueldre, C., Leclercq, Y., Phillips, C., Luxen, A., Vandewalle, G., Vogels, R., Maquet, P. & Balteau, E. 2013. The impact of visual perceptual learning on sleep and local slow-wave initiation. J Neurosci, 33, 3323-31.

Memories are consolidated during sleep by two apparently antagonistic processes: reinforcement of memory-specific cortical interactions and homeostatic reduction in synaptic efficiency. Using functional magnetic resonance imaging, we assessed whether episodic memories are processed during sleep by either or both mechanisms, by comparing recollection before and after sleep. We probed whether long-term potentiation (LTP) influences these processes by contrasting 2 groups of individuals prospectively recruited based on brain-derived neurotrophic factor rs6265 (Val66Met) polymorphism. Between immediate retrieval and delayed testing scheduled after sleep, responses to recollection increased significantly more in Val/Val individuals than in Met carriers in parietal and occipital areas not previously engaged in retrieval, consistent with 'systems-level consolidation'. Responses also increased differentially between allelic groups in regions already activated before sleep, but only in proportion to slow oscillation power, in keeping with 'synaptic downscaling'. Episodic memories seem processed at both synaptic and systemic levels during sleep by mechanisms involving LTP (see Figure 4).

Mascetti, L., Foret, A., Schrouff, J., Muto, V., Dideberg, V., Balteau, E., Degueldre, C., Phillips, C., Luxen, A., Collette, F., Bours, V. & Maquet, P. 2013. Concurrent synaptic and systems memory consolidation during sleep. J Neurosci, 33, 10182-90.

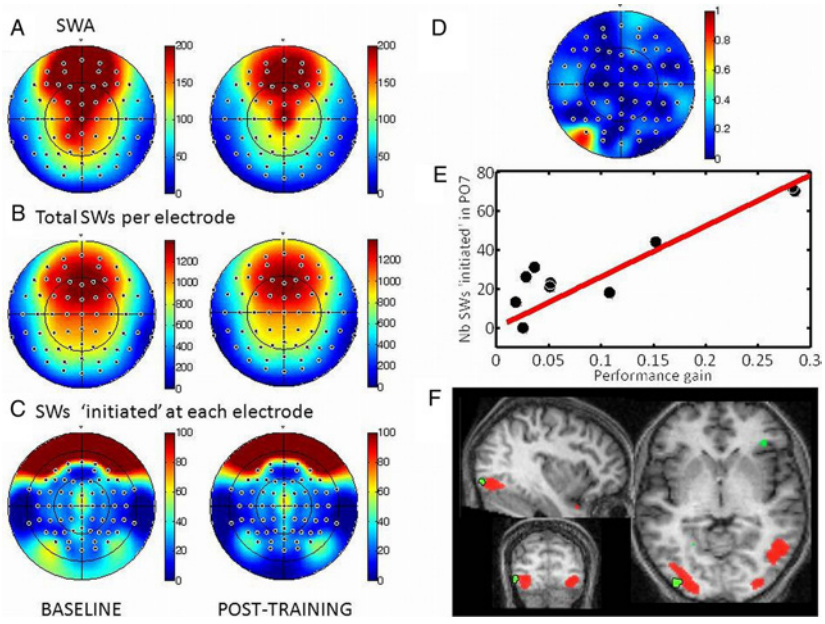

Figure 3. EEG and fMRI results. A, Scalp distribution of SWA during the first NREM cycle of baseline (left) and posttraining (right) nights. B, Scalp distribution of the number of SWs crossing each electrode position during the first NREM cycle of baseline (left) and posttraining (right) nights. C, Scalp distribution of the number of SWs initiated at each electrode position during the first NREM cycle of baseline (left) and posttraining (right) nights. D, Scalp distribution of squared correlation coefficients (r^2) between the number of SWs initiated at each electrode position during the first NREM cycle of posttraining night and the overnight gain in performance. E, Correlation between the number of SWs initiated at PO7 during the first NREM cycle of posttraining night and the overnight gain in performance. F, Coregistration of the current sources of SWs initiated at PO7 during the first NREM cycle of posttraining night (red) and the area showing an overnight increase in learning response during fMRI testing (green) averaged across all subjects.

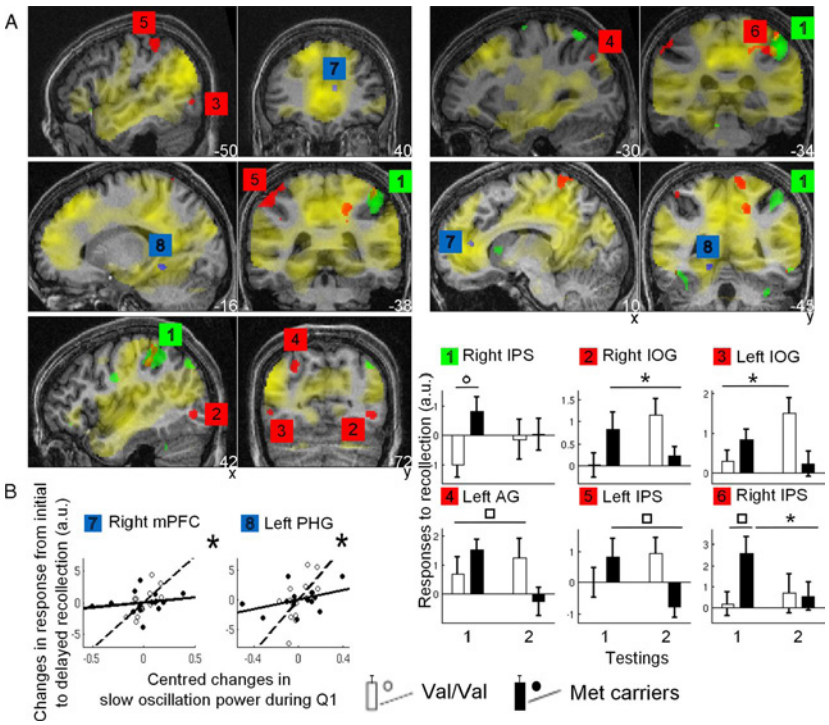

*Figure 4. fMRI results. A, Yellow, Brain areas recruited during initial recollection in both groups. Green, Larger responses in Met carriers than in Val/Val during initial recollection. Red, Larger changes in response from initial to delayed recollection (Val/Val > Met carriers). Blue, Areas in which changes in Val/Val are proportional to the changes in SOP from baseline to test night. Functional results are displayed at $p_{uncorrected} < 0.001$, except for initial testing displayed at $p_{uncorrected} < 0.05$ (to show all areas likely to be involved in early memory formation), over a typical individual MR image. B, Parameter estimates (mean ± SEM) of brain areas labeled as in A. The two left bars pertain to initial testing (testing 1), and the two right bars pertain to delayed testing (testing 2). White bars, Val/Val; black bars, Met carriers. °$p_{FWE\text{-}whole\ brain} < 0.05$ (1); *$p_{SVC} < 0.05$ [2, 3, 6 (right bar), 7, 8]; □$p_{uncorrected} < 0.001$ [4, 5, 6 (left bar)]. Scatter plots for areas 7 and 8 represent the regression between individual activity estimates and changes in SOP during Q1, assessed as the first eigenvariate of the singular decomposition of significant changes in both groups in sleep EEG power between baseline and test night. a.u., Arbitrary units. Open circles, dotted lines, Val/Val; filled circles, solid lines, Met carriers.*

The regulation of human sleep and wakefulness

At any point in time, cognitive performance results from the interaction between two opposing signals: the sleep pressure accrued during wakefulness and circadian rhythm. For several years, we investigate the neural correlates of this interaction in different populations.

We published a follow up study of our Science paper on extreme chronotypes, using a different cognitive task. Human morning and evening chronotypes differ in their preferred timing for sleep and wakefulness, as well as in optimal daytime periods to cope with cognitive challenges. Recent evidence suggests that these preferences are not a simple by-product of socio-professional timing constraints, but can be driven by inter-individual differences in the expression of circadian and homeostatic sleep-wake promoting signals. Chronotypes thus constitute a unique tool to access the interplay between those processes under normally entrained day-night conditions, and to investigate how they impinge onto higher cognitive control processes. Using functional magnetic resonance imaging (fMRI), we assessed the influence of chronotype and time-of-day on conflict processing-related cerebral activity throughout a normal waking day. Sixteen morning and 15 evening types were recorded at two individually adapted time points (1.5 versus 10.5 hours spent awake) while performing the Stroop paradigm. Results show that interference-related hemodynamic responses are maintained or even increased in evening types from the subjective morning to the subjective evening in a set of brain areas playing a pivotal role in successful inhibitory functioning, whereas they decreased in morning types under the same conditions.

Furthermore, during the evening hours, activity in a posterior hypothalamic region putatively involved in sleep-wake regulation correlated in a chronotype-specific manner with slow wave activity at the beginning of the night, an index of accumulated homeostatic sleep pressure. These results shed light into the cerebral mechanisms underlying inter-individual differences of higher-order cognitive state maintenance under normally entrained day-night conditions.

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

In 2011, we conducted the first-ever fMRI study in normal volunteers submitted to a 42-hour constant routine, during which 13 fMRI sessions were acquired. The data are still being analyzed. During pilot studies, we probed the usefulness of various cognitive tasks, to be used in this protocol. The Attention Network Test (ANT) was considered because it is deemed to assess the alerting, orientating and executive components of human attention. Capitalizing on the opportunity to investigate three facets of attention in a single task, we used functional magnetic resonance imaging (fMRI) to assess the effect of sleep deprivation (SD) on brain responses associated with the three attentional components elicited by the ANT. Twelve healthy volunteers were scanned in two conditions 1 week apart, after a normal night of sleep (rested wakefulness, RW) or after one night of total sleep deprivation. Sleep deprivation was associated with a global increase in reaction times, which did not affect specifically any of the three attention effects. Brain responses associated with the alerting effect did not differ between RW and SD. Higher-order attention components (orientating and conflict effects) were associated with significantly larger thalamic responses during SD than during RW. These results suggest that SD influences different components of human attention non-selectively, through mechanisms that might either affect centrencephalic structures maintaining vigilance or ubiquitously perturb neuronal function. Compensatory responses can counter these effects transiently by recruiting thalamic responses, thereby supporting thalamocortical function.

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

Non visual responses to light influence brain cognitive responses

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.

We also conducted an fMRI study which is based on physical properties of melanopsin, the photopigment that conveys the non-image forming visual responses.

Light stimulate alertness, cognition and sleep-wake regulation in humans. This non-image-forming (NIF) impact of light is mediated through a photoreceptor system that is maximally sensitive to blue light, presumably through the recruitment of intrinsically-photosensitive retinal ganglion cells (ipRGCs) that express the photopigment melanopsin. The direct implication of ipRGCs in sleep and wakefulness regulation is established in nocturnal rodents. In humans, evidence for their involvement is indirect, due to the difficulty to selectively silence or enhance contributions of ipRGCs, rods and/or cones. Therefore, the contribution of ipRGCs to the impact of light on human alertness and cognition remains to be established.

When capturing photons, chromophore of rod and cone photopigments go from a photosensitive to photoinsensitive conformation, which is responsible for phototransduction. Enzymatic activity within the retinal pigment epithelium is then required for chromophore regeneration. By contrast, melanopsin is a dual-state (bistable ?) photopigment switching between two photosensitive conformations, and light seems to drive both phototransduction and at least part of chromophore regeneration. Recent *in vivo* rodents and human data suggest that exposure to longer wavelength light (590-620nm; orange-red) triggers chromophore regeneration and increases subsequent photosensitivity of ipRGCs. Conversely, exposure to shorter wavelength light (~480nm; blue) favors phototransduction and decreases subsequent ipRGC photosensitivity. The present study aimed at establishing ipRGCs influence on human cognitive brain function based on this photic history hypothesis of melanopsin dual-states. We hypothesized that the impact of a test light on cognitive brain responses would be increased, decreased or intermediate following prior exposure to longer, shorter or intermediate wavelength light, respectively.

Sixteen participants took part in a balanced crossover-design comprising 3 identical 15min fMRI recordings, which included blocks of auditory 0- and 3-back tasks while a test light (515nm) was administered with a pseudo-randomly changing irradiance level. Responses to the working memory task (3-back) were contrasted to responses to simple letter detection task (0-back) to isolate executive brain responses. Each recording was preceded by 70 minutes of darkness allowing complete dark adaptation of rods and cones, which were preceded by 10min exposure to shorter (461nm), intermediate (515nm) or longer (589nm) wavelength light.

Despite identical scanning conditions during fMRI sessions, executive responses significantly differed depending on prior light history. Relative to prior blue condition, prior orange light exposure significantly enhanced the modulation of executive responses by test light in bilateral superior and inferior dorsolateral prefrontal cortex (DLPFC) and in the left ventrolateral prefrontal cortex (VLPFC). These frontal areas have been implicated in various levels of executive control. Likewise, relative to prior blue condition, prior orange light exposure significantly increased test light impact in pulvinar responses, a region essential

to arousal and cognition regulation that plays a key role in mediating the impact of light on alertness and cognition. Additionally, similar impacts of prior orange light were detected within the amygdala, fusiform gyri, substantia nigra and cerebellum. These results emphasize the importance of light history for human alertness and cognition and demonstrate that exposure to longer wavelength light enhances the subsequent impact of light on cognitive brain function.

Further support to this concept comes from the results of prior green light that, relative to prior blue exposure, resulted in an increased test light impact in the same left VLPFC location as for prior orange to prior blue light. However, compared with prior green light, prior orange light exposure still increased to a significantly larger extent the impact of test light on the amygdala and cerebellum. By contrast, the comparisons between prior shorter wavelength vs. prior longer wavelength light (prior green>orange; prior blue>orange; prior blue>green) revealed no significant differences.

Given the strict experimental protocol, the observed differences are in line with the prior history hypothesis of melanopsin dual-states, and provide experimental evidence that light history affects higher cognitive functions through melanopsin-expressing ipRGCs. They strongly suggest a cognitive role for melanopsin-expressing ipRGCs, which may confer a “photic memory” to human cognition.

These results are submitted to publication.

Final report of the research group of

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Unravelling the roles of lysine acetylation in neural development

Backround

The aim of our project 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). 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. Although this PTM has mainly been 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.

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 our project is to uncover new proteins that undergo lysine acetylation and decipher how their acetylation contribute to cerebral cortical neurogenesis (Aim 1) or inner ear development (Aim 2). For this purpose we focussed our work on the Elongator complex, which is composed of 6 subunits (Elp1-6) and is expressed both in the nucleus and the cytoplasm where it plays multiple functions. Although the complex promotes acetylation of histones in the nucleus to contributes to transcript elongation, it promotes exocytosis and tRNA modification as well as cytoskeletal modifications in cortical neurons, as recently shown by our laboratory (Creppe et al., 2010; Creppe et al., 2009; Nguyen et al., 2010).

The following report summarizes the work performed during the past three years 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). 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.

Elongator controls microtubule-dependent activity in neurons

We recently showed that the acetylation of tubulin is required for proper migration and differentiation of cortical projection neurons. Indeed, such modification of the MTs contributes to MT-dependent transport by promoting the anchoring of some molecular motors.

Motors are important for the transport of various cell components in neurons such as cytoskeleton elements, synaptic vesicles or mitochondria, among others. To ensure the transport, kinesins are responsible of the anterograde transport towards the plus-end of microtubules and the periphery of the axon (the synapse); dyneins moves in the opposite orientation towards the minus-end of microtubules and the cell soma, this represents the retrograde transport. Importantly, molecular transport underlies the regulation of cell shape remodeling during neuritogenesis and neuronal migration and may be partly disrupted in neurons that lack Elongator activity. We thus tested the effect of Elongator depletion on the retrograde and anterograde transport of specific cargo proteins in both mouse cortical neurons in culture (in process) and third instar drosophila larva motoneurons *in vivo*. We analysed defects resulting from loss of function of Elp3 an/or aTAT1/2 (another potent promoter of alpha-tubulin acetylation (Akella et al., 2010)) to 1/assess individual transport phenotypes in drosophila, 2/ combine invalidation of both genes to induce a more efficient depletion of acetylation of MT.

During Drosophila's life cycle, and more precisely at the larval stage, the cuticle is transparent and enables imaging of moving GFP-tagged cargoes. Moreover, in Drosophila two motors achieve transport, one kinesin and one dynein. We took advantage of the binary system Gal4-UAS, to knockdown Elp3 and/or aTAT1/2 together with the expression of GFP-tagged synaptotagmin, to label vesicles, or GFP-tagged mitochondria in larval motoneurons. We further recorded their movements by time-lapse confocal microscopy. Our preliminary results showed that depletion of Elp3 and/or aTAT1/2 did not affect the average speed of moving vesicles either in antero- or retrograde direction. However, depletion of Elp3 led to a reduced time spent in pauses, meaning that vesicles tend to pause less than in controls. On the other hand, the percentage of moving vesicles was only reduced after aTAT1 and/or aTAT2 depletion. The axonal transport of mitochondria was also partly impaired after Elp3 depletion. While, the mean speed of moving mitochondria remained unchanged, the depletion of Elp3 led to a reduced

time spent in pause. In addition, depletion of aTAT1 and aTAT2 led to a reduced number of moving mitochondria. Altogether, our preliminary results suggest that Elp3 control some aspects of the MT-dependent transport of vesicles and organelles along axons. It is worth noting that Elp3 and aTA1/2 do not play the same role in MT-dependent molecular transport, suggesting different activities towards tubulin and maybe cargoes. Those results will be compared to those obtained with hiPS cell-derived neurons from Familial Dysautonomia patients (Elp1 mutant) (collaboration with L. Studer, NY, USA and G. Lee, Baltimore, USA). This information will likely contribute to better understand some aspects of the pathological mechanisms that underlie Familial Dysautonomia.

Elongator promotes the fine regulation of cortical interneuron migration

To investigate the role(s) of Elongator in cortical interneuron migration, we used a conditional Elp3 lox/lox; Dlx5,6 Cre-GFP (Elp3 conditional knockout mouse line in interneurons, or Elp3 cKO-in) mouse line newly generated in our laboratory. The dynamic analysis of interneurons migration and cell shape transformations were performed by time-lapse experiments on Elp3 cKO-in embryos or controls at E13.5 MGE explants cultured on monolayer of WT cortical cells. After 24 hours of culture, control GFP+ interneurons that have migrated out of MGE explants showed a polarized morphology with branched leading process. Our data indicate that interneurons that lack of Elp3 expression showed a significant decrease of migration velocity as well as frequency and amplitude of nuclear translocations. We also observed that several Elp3 cKO-in migrating interneurons underwent swelling formation with a shorter mean distance between the nucleus and the swelling (bulge). Moreover, live imaging also indicated that the formation and division of growth cones (that underlie the production of new branches) were also less frequent in Elp3 cKO-in interneurons. In addition, time-lapse analyses performed on 300 µm organotypic slices showed similar results with a significant decrease of migration velocity, migration persistence, as well as a reduction of both frequency and amplitude of nuclear translocations. Immunolabelings performed on E12.5 and E14.5 embryo forebrain sections showed that loss of Elp3 expression affects the cellular shape and reduces the number of interneurons that entered into the cortex after crossing the cortico-striatal boundary. Furthermore, analyses in lateral, dorso-lateral and dorsal fields divided in 10 equal bins revealed that both the marginal and the subventricular zone (SVZ) cellular streams were significantly affected upon Elp3 depletion. Collectively, our data suggest that Elp3 is required for proper nucleokinesis and branching dynamics of migrating cortical interneurons in vivo. To decipher how Elp3 controls tangential migration, we are currently performing rescue experiments (focal electroporation of specific plasmids followed by time-lapse recordings on cultured brain slices) with either Elp3 full length or its mutant forms that lack functional domains (the Histone acetyl transferase (HAT) domain and/or the DNA methyltransferases (SAM) domain). Elongator acts as a KAT but in contrast to observations made earlier with projection neurons, we showed that α-tubulin was properly acetylated upon conditional Elp3 removal in interneurons. Thus we postulated that the migration dynamics and branching activity of cortical interneurons could be independent of the activity of Elongator towards α-tubulin. We are thus currently trying to identify new Elp3 targets involved in tangential migration that could be acetylated. For this purpose we outsourced “acetylscans” on micro-dissected forebrain tissue isolated from E14.5 Elp3 cKO or wild type embryos (*Cell Signaling*). This approach should help us identifying the molecular mechanisms triggered by Elongator to control the tangential migration of interneurons in the developing cerebral cortex.

Elongator controls the specification of cortical progenitors

We previously described the expression of Elongator subunits in cortical progenitors (Creppe et al., 2009), but for technical limitations we couldn’t to decipher its function in cycling progenitors. Thus we engineered a new mouse model by crossing Elp3lox/ox mice with Cre reporter mice expressing

a FoxG1:Cre (Elp3cKO) to allow the analysis of Elongator's functions in cortical progenitors. Elp3cKO embryos suffered from microcephaly and showed a strong reduction in both, size and thickness of the cortex at E14.5 as well as after birth. The Elp3 cKO pups survived until day postnatal (P) 25 at most.

We further performed complementary experiments to analyse the cortical phenotype of Elp3cKO mouse embryos and assessed cell survival, proliferation, and specification and progenitor differentiation into neurons. Our results showed that the survival of progenitors and neurons was not affected upon depletion of Elp3 as the number of activated-caspase3 positive cells was not significantly increased in Elp3cKO embryos. The apical progenitor population (that expresses *Sox2* and *Pax6*) was not impaired by the loss of Elp3. This was confirmed by immunolabelings to measure their proliferation rate (number of mitosis: phospho-histone H3 labelling; the number of cells in S phase: BrdU incorporation one hour before sacrifice). Interestingly, we observed a strong reduction of the number of Tbr2-expressing basal progenitors that populate the subventricular zone, together with a proportional reduction of basal mitoses. These cells arise from neurogenic division of apical progenitors and are responsible for the production of the great majority of cortical postmitotic neurons that populate all cortical layers. Consequently we observed a severe reduction of number of neurons expressing β3-tubulin in the Elp3cKO cortex. This reduction was observed in all cortical layers, as shown by in situ hybridization for laminar markers in cryosections from P0 cortices. Our current hypothesis is that part of the cortical phenotype observed in Elp3cKO embryos could result from specific production defect of basal progenitors from apical progenitors. In order to evaluate this transition step, we labelled apical progenitors *in vivo* with GFP using in utero electroporation of E13.5 Elp3lox/lox embryos with plasmids expressing GFP or Cre-GFP. Our preliminary results showed a reduction of the number of cells expressing both GFP and Tbr2 when Cre is expressed. This is very interesting and mimics the brain phenotype of Tbr2cKO embryos. Although Tbr2 transcription was not affected by the loss of Elp3 (evaluated by qRT-PCR and *in situ* hybridisation), we observed a severe reduction of the Tbr2 protein expression level in the developing cortical wall of Elp3 cKO (measured by western blot and highlighted by immunolabeling). We are currently combining multiple approaches to identify the molecular pathway disrupted upon Elp3 depletion that lead to apical-to-basal progenitor transition defects. The reduction of Tbr2 protein expression may either result from increased Tbr2 protein degradation (competition between acetylation and ubiquitination for shared targeted lysines) or from a specific translation defect of Tbr2 mRNA. To evaluate the translation level of Tbr2 upon Elp3 removal, we set up a collaboration with S. Leidel (Max-Planck, Munster) who will perform ribosome profiling. For this purpose, we will provide him with RNA extracted from cortical cells purified after FACS-isolation. Cortical progenitors of Sox2CreRT2;RosaSTOPYFP;Elp3lox/lox E14.5 embryos will be isolated two days after tamoxifen injections (i.p. in pregnant dam) to allow a specific removal of Elp3 in most apical progenitors that will additionally express GFP. After extraction, nuclease digestion will allow isolation of the pool of mRNA that is actively translated, and the comparison with RNA sequencing library after normalisation (deep sequencing on the same material to measure the absolute abundance of transcripts in apical progenitors) will allow comparison of Tbr2 translation rates between Elp3cKO embryos and corresponding WT embryos. In addition, we are addressing the degradation level of Tbr2 when Elp3 is knockdown in cultured cells. We are currently working with HepG2 cells that express Tbr2 endogenously. Proteasome inhibition (MG132 treatment) promoted Tbr2 accumulation in these cells *in vitro*. Conversely, treatment of these cells with cycloheximide (pharmacological blockade of mRNA translation) resulted in a strong reduction of Tbr2 protein expression. Using these two drugs, we are investigating the degradation rate of Tbr2 in WT Elp3-depleted HepG2 cells.

Finally, additional results showed an accelerated interkinetic nuclear movement (INM) of apical progenitor nuclei in Elp3cKO embryos. Thus, in addition to inducing direct molecular defects (regulating

protein translation or stability) in apical progenitor, the loss of Elp3 may indirectly mediate apical to basal transition by disrupting INM. To evaluate INM in E14 mouse embryos, we injected BrdU in pregnant mice at different timing before sacrifice (1h, 4h and 6h). We further assessed the position of the BrdU-positive nuclei in the cortex. At these three different injection times, we observed the accumulation of apically positioned nuclei in Elp3cKO embryos compared to WT littermates, consistent with an increased speed of the INM. Moreover, nucleus showed a more elongated shape in Elp3cKO embryos, suggesting that these nuclei undergo stronger tension forces that may reflect accelerated INM

One of the candidate Elp3-target proteins is connexin-43 (cx-43). We found that this connexin is enriched in the developing cerebral cortex and is massively acetylated. Western blot analyses performed on cortical extracts from Elp3cKO E12 mouse embryos showed a dramatic reduction of the level of acetylation of cx-43. In addition, co-immunoprecipitation assays with cortical tissue extracts demonstrated an interaction between the connexin and Elp1, the scaffold subunit of the Elongator complex. Therefore cx-43 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 now assessing the putative role of cx-43 acetylation in corticogenesis, focussing on the control of the interkinetic nuclear movement (INM).

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

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

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

Using the newly created mouse line Elp3loxp/loxp, we generated FoxG1-cre conditional Elp3 knockout mice (referred to as Elp3 cKO) allowing the deletion of Elp3 in the entire otocyst at ≈E8.5. Although they were viable, these mice exhibited balance-related behavioral phenotype characterized by a tilted position of the head, circling movements, and a marked tendency to walk backwards when placed outside their cages. In addition, in the tail-hanging reflex, which normally induces a forelimb extension

to reach the ground, they tended to bend ventrally and curl up their tail. More recently, we showed that these mice are deaf. Indeed, the auditory brainstem response (ABR) thresholds were elevated in Elp3 cKO mice as compared to wild-type littermates. We also analyzed Elp3 cKO mice at the cellular level both in the sensory epithelium and the cochleo-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. We are currently testing whether this cell death is maintained later during development. There is also a conspicuous decrease of the number of fibers that innervate hair cells. In addition, numerous remaining fibers present aberrant projections towards inner hair cells. More recently, we showed that the number of synaptic ribbons, quantified after immunostainings using anti-Ctbp2/Ribeye antibody is significantly decreased at the level of inner hair cells. Ongoing work is focused on further characterization of spiral ganglion neuron-hair cell synapse using co-labelling with Ctbp2 and Glur2/3 (a post synaptic marker) and transmission electron microscopy preparations. RNA-seq study has recently been performed in E14.5 wild-type and Elp3-cKO cochleae and showed an unexpected increased of hair cell-specific mRNAs, such as gfi1, Atoh1 and pou4f3. This potential precocious hair cell differentiation will be verified by in situ hybridization on E14.5 cochlear explants. If we confirmed these results, we will determine if this premature hair cell differentiation is caused by earlier cell cycle exit of progenitor cells. We also showed that the length of the cochlea is reduced in cKO animals, which reinforced this hypothesis.

Altogether, these results indicate that Elp3 seems to be involved in neuronal survival, neurite guidance and hair cell differentiation in the cochlea. We will further try to identify the mechanisms underlying this phenotype. Transcriptional Elp3 targets will be selected based on the decrease of mRNA level in Elp3-cKO in the RNA-seq experiment. At the protein level, a candidate approach will also be undertaken and we will focus on Ctpb2 as previously demonstrated as an acetylable protein (Zhao et al., 2006).

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Final report of the research group of

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

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

The major aims of our project are to dissect out the distinct properties and identify the precise role of striatal neuronal populations and genes in motor control, movement disorders 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 main achievements obtained on the 2011-2013 period thanks to the support from QEMF are summarized below.

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

1.1 Effects of specific ablations D_1R-striatonigral and D_2R-striatopallidal MSN in distinct dorsal striatum subregions.

Using a transgenic $A_{2A}R$-Cre mouse strain that we previously developed (Durieux et al., 2009,2011) and which allowed to conditionally target the expression of a human diphteria toxin receptor (DTR) in striatopallidal neurons, our previous works showed that selective ablations of these D_2R-MSN can be performed in different restricted functional areas of the striatum and produce specific behavioural alterations. Indeed, D_2R striatopallidal MSN ablation in the entire striatum produces permanent hyperlocomotion while restricted ablation in the ventral striatum resulted in an increase in drug reinforcement and in its much longer persistence, demonstrating that D_2R striatopallidal MSN exert inhibitory functions on both locomotor control and drug reward process (Durieux et al., 2009,2011).

We have further developed a parallel model allowing specific removal of the D1R striatonigral MSN (Durieux et al., 2012) by using a similar strategy and a Drd1a-Cre mice strain (Gong et al., 2007) targeting striatonigral neurons. Analysis of the resulting mice in locomotor paradigms indicate that 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., 2012). When compared with the persistent hyperactivity (about 400%) induced by a specific ablation of D2R striatopallidal MSN (Durieux et al., 2009,2011), 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., 2012). 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., 2012).

Furthermore, the dorsal striatum is subdivided into the dorsolateral striatum, DLS, corresponding to the primate putamen, predominantly innervated by the sensorimotor cortex and the dorsomedial striatum, DMS, homologous to primate caudate nucleus, receiving projections from prefrontal and other association cortices (Graybiel, 2008). The DMS is more engaged during initial stages of motor skill learning, when the task is more dependent on attention and susceptible to interference (Luft and Buitrago, 2005) while the DLS is required for progressive skill automatization and habit learning (Yin et al., 2004, 2009) but the specific involvement in motor learning phases of striatopallidal and striatonigral neurons in the different striatal sectors was completely unknown. Elimination of D_1R-striatonigral neurons in the DMS induced a reduction in ambulation while, in contrast, DMS D_2R-striatopallidal neuron ablated mice displayed hyperlocomotion. This showed that the modulatory influence on locomotion observed following full ablations was recapitulated in DMS-restricted ablations, indicating that associative striatum area exerts a MSN population-dependent control over spontaneous locomotion. (Durieux et al., 2012). 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., 2012). 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 targets of pharmacological treatments in movement disorders as Parkinson's disease or in schizophrenia and are also the targets of drugs of abuse but the involvement of D1R striatonigral and D2R striatopallidal MSN in the DMS and DLS, respectively, was unknown.

Treatment of schizophrenia positive symptoms with typical neuroleptic drugs is often associated with motor side effects such as catalepsy. We evaluated involvement of each neuronal population in motor responses to neuroleptic drugs and showed that only D_2R-striatopallidal neuron removal selectively in the associative striatum completely abolished haloperidol–induced catalepsy, indicating that D_2R-antagonism in striatopallidal neurons of the associative striatum (DMS) is critical for the motor effects of haloperidol (Durieux et al., 2012).

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 psychostimulants and showed that DMS striatopallidal D_2R-neurons necessarily contribute to the development of behavioural sensitization to amphetamine (Durieux et al., 2012). Therefore, although numerous studies supported a specific role of the ventral striatum as the main striatal region contributing to the development of psychostimulant sensitization, our results strongly pointed to a necessary contribution of DMS D2R-neurons in amphetamine sensitization.

Altogether, our results provided 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.

1.2 Specific optogenetic control of D_1R-striatonigral and D_2R-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. To circumvent these issues, during the frame of this project, we therefore started to develop optogenetics, that is based on the use of light-activatable proteins ("opto-") encoded in DNA ("-genetic") to reversibly modulate in physiological millisecond timescale, *in vivo* or *ex vivo,* the activity of genetically targeted neuronal populations in rodents. We first examined the feasibility of the technic *ex vivo*. Adeno-associated virus (AAV), in which expression of Channelrhododopsin-2 (ChR2) cation channel, fused with eYFP, is dependent upon Cre-recombination has been stereotactically injected for transfection into the striatum of A_{2A}R-Cre mice (see above and Durieux et al., 2009,2012). Experiments combining perforated patch clamp recording and combined to optical stimulation of ChR2-expressing D2R MSN 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. Optical stimulation of these neurons with a blue light (470 nm) resulted in fast inward currents when recorded in voltage clamp and in the evocation of action potentials when recorded in current clamp (Fig 1). The validity of the technique was also demonstrated by the high-frequency reliability of the light-induced action potentials in trains. The same experiments have been carried out in Drd1a-Cre mice.

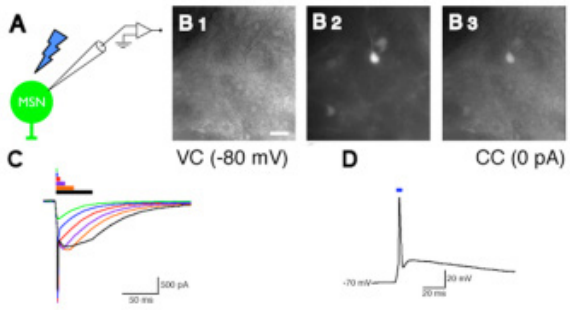

Figure 1: Optogenetic control of D2R-striatopallidal MSN in slices from Adora2-Cre mice injected with ChR2-YFP AAV. (A) MSN recording and optical stimulation. (B1) Recorded MSN in transmission and (B2) fluorescence. (C) Currents evoked by light pulses of increasing durations. (D) Action potential (AP) evoked by a 5ms light pulse.

Based on our ex vivo recordings, we chosen a specific pulse frequency and duration and performed first experiments to validate the opotogenetic technique in vivo. We showed the induction of contralateral and ipsilateral rotations in Adora2a- and Drd1a-Cre mice (circling behaviour), respectively, after transfection with AAV1 ChR2 in DLS and unilateral light stimulation (20Hz, 5ms pulse) (Fig. 2).

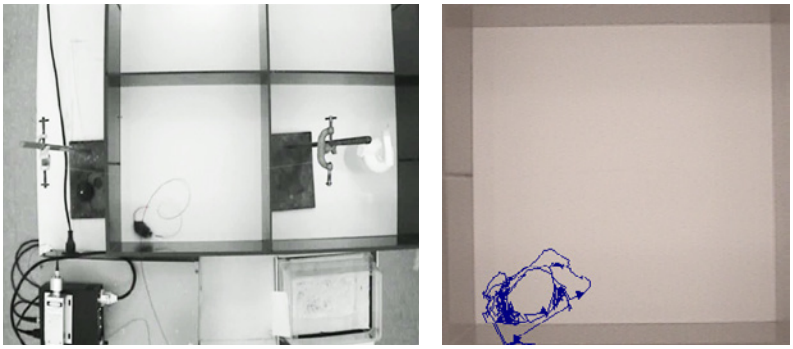

Figure 2: In-vivo optogenetic control of striatonigral D1R-MSN in right DLS from Drd1-Cre mice injected with ChR2-YFP AAV1. Left: In vivo optogenetic openfield setup. Right: Tracking (1 min.) of contralateral rotations (left rotation) induced by light stimulation at 20Hz

To circumvent the potential variability due to stereotaxic injections, as an alternative, we also started the use of mice allowing the conditional expression of ChR2 using the Cre/lox strategy (ROSA26-lox-Stop-lox-ChR2-EYFP) (Madisen et al., 2012). These “floxed” mice have been crossed with the Drd1-Cre or Adora2a-Cre mice in order to express the opsins in the desired cell population. We already obtained ChR2-EYFP-expressing mice in D2R-MSN and demonstrated in slices, the efficiency of optical stimulation to evoke AP trains in Adora2a-expressing ChR2 neurons (Fig. 3). In this strategy, the regional selectivity (DMS, DLS, NAc) will be provided in vivo by the settings of the illumination device.

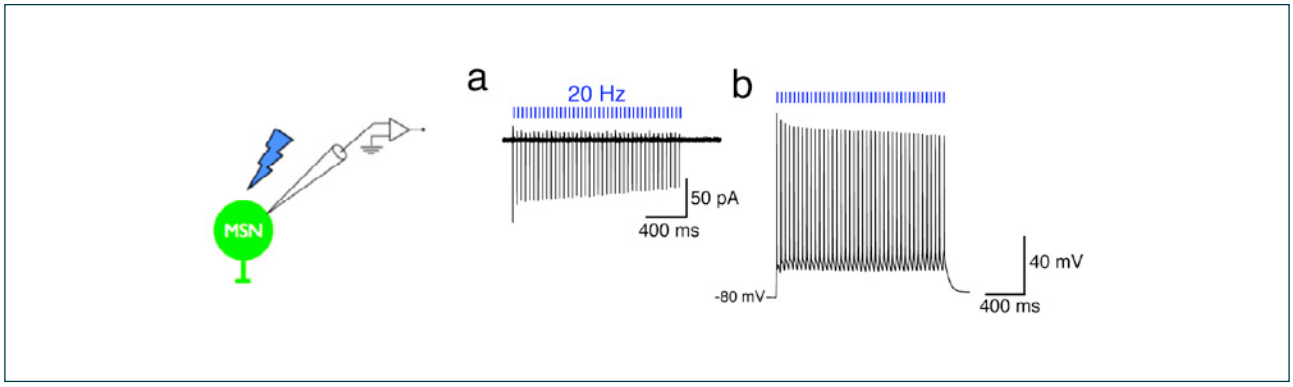

Figure 3: Optogenetic control of a striatopallidal D2R-MSN in slice from an Adora2-Cre x ROSA26-lox-Stop-lox-ChR2-EYFP mouse. (a) Currents and (b) Action potentials evoked by 20 Hz trains of 5ms light pulses

As a perspective, using these mice models, we will produce reversible optogenetic stimulation or inhibition of D1R- or D2R-MSN in different functional striatal areas (DMS, DLS and NAc) during different behavioural tasks and restricted learning phases. To identify the populations involved in neuroadaptation and behavioural deficits in Parkinson's disease and drug addiction, and the potential to restore normal behaviours, such paradigms will be conducted in mice modeled for these diseases.

1.3 Specific inactivation of NR1 in striatopallidal and striatonigral neurons

Neuroadaptation and more specifically synaptic plasticity involve several important neurotransmitter receptors and intracellular signalling cascades. Among the involved receptors, the Ca^{2+} permeable glutamate NMDA receptor is a central and initial player. This has been firmly demonstrated at different excitatory synapses such as in the hippocampus (Tsien et al., 1996). 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 (Schiffmann et al., 2007). 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. A recent publication demonstrated that these NR1 floxed mice (Tsien et al., 1996) have not a yield of cre recombination of 100% and that other strain of NR1 floxed mice (Dang et al., 2006) has a better recombination's yield because the LoxP sites are closer (Belforte et al., 2010). We have established collaboration with Prof. Li and have crossed his NR1 floxed mice with our A_{2A}R-Cre/+ mice to obtain new A_{2A}R-Cre/+ NR1$^{f/f}$ as well as with Drd1a-Cre/+ mice to obtain Drd1a-Cre/+ NR1$^{f/f}$ and therefore cell-specific NR1 inactivation in both D_1R striatonigral and D_2R striatopallidal neurons. In addition, to increase our chance of full recombination, we developed a strategy allowing to generate A_{2A}R-Cre/+ NR1$^{delta/f}$ and Drd1a-Cre/+ NR1$^{delta/f}$. Finally, in order to allow the identification of neurons deficient in NR1 in brain slices for patch clamp recordings, these mice were further crossed with reporter mice (LoxP-Stop-LoxP-YFP) leading to the expression of *Yellow Fluorescent Protein* (YFP) in recombined neurons. We obtained first series of mice and showed that in a significant percentage of YFP-labelled neurons, there is a total absence of the NMDA receptor-mediated component of the EPSC (Fig. 4).

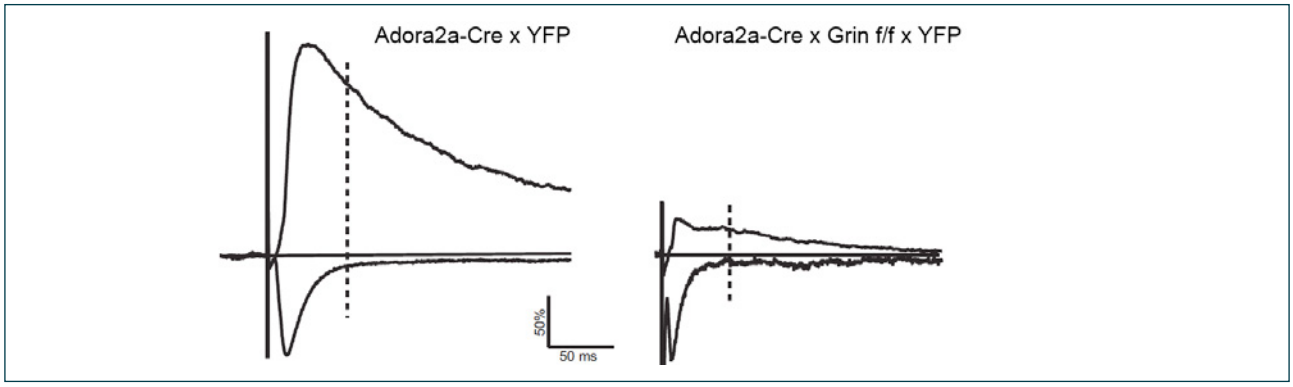

Figure 4: Absence of the NMDA-component of evoked EPSCs in D2R-MSN in slice from Adora2a-Cre x Grin f/f x YFP as compared to a control D2R-MSN. In each cell, EPSCs were evoked at -70 mV and +40 mV.

First series of behavioural analysis have been performed on these new A_{2A}R-Cre/+ $NR1^{f/f}$ mice. As a perspective, 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, …).

1.4 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 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. We succeed in isolating green (striatopallidal) and red (striatonigral) neurons from individual mice with a very good rate of enrichment. RNA amplification and identification was performed to validate the differential expression of some known genes (i.e. enkephalin, substance P, A_{2A} receptor) showing that we have an enrichment of more than 90% (Ena et al., 2013). Gene profiles of these neurons have been obtained by micro-arrays and showed 248 striatopallidal neuron specific genes and 493 striatonigral neuron specific genes (> 2 fold differential expression). Although some genes were already known to be highly restricted to one of these subpopulations, several striatonigral neuron-specific or striatopallidal neuron-specific genes that showed a relative expression of several tens to hundred fold, were not known to be selectively expressed or even not known to be expressed in the striatum (Ena et al., 2013). This differential gene expression has been validated by using different techniques for a dozen of genes. Among these genes, we have selected a series of striatopallidal neuron-specific genes as NTe5, RGS5, GuaCy13A, Adk that exhibit both a high differential expression and a putative physiological relevance for further analysis using different knock-down strategies.

We validated the specific expression of the nucleotidase NTe5 in striatopallidal neurons by RT-PCR, enzymatic activity histochemistry and immunocytochemistry (Ena et al., 2013). NTe5 is an ecto-enzyme known to produce extracellular adenosine from 5'-AMP Since adenosine, acting at A_1 and A_{2A} receptors, is an important neuromodulator in the central nervous system and more specifically in the striatum where A_{2A} receptors are highly enriched and play crucial functional roles (Schiffmann et al., 2007), such a specific expression may have important functional impact. To assess for the involvement of this nucleotidase in behaviour, we used a global NTe5 knockout mice strain and develop striatal or striatopallidal neuron selective knock-down strategies using lentivirus-mediated small hairpin RNA. In order to analyze a striatal-dependent learning task, mice were trained in a motor skill learning task on

an accelerating rotarod. This behavioral analysis on both striatal and striatopallidal neuron knock-down models as well as on the knockout mice showed that mice behave less accurately, demonstrating therefore the implication of this enzyme in striatopallidal neurons in motor learning (Ena et al., 2013).

To test if this deficit results from a decrease of A_{2A} receptor activation as a consequence of the absence of the nucleotidase and decrease in extracellular adenosine, we quantified the catalepsy induced by the injection of the dopamine D_2 antagonist haloperidol, a behaviour dependent on the tonic activation of A_{2A} receptor. We showed that the cataleptic response to haloperidol was significantly decreased in the KO mice compared to wild-type (Ena et al., 2013).

RGS5 is a member of the large family of Regulators of G-protein Signalling (RGS) that are negative regulators of signalling cascades induced by proteins G. Although its role has been examined in blood vessels, very few data reported the involvement of RGS5 in brain functions apart from the fact that association study has shown a possible involvement of RGS5 in the schizophrenia symptom severity and that striatal RGS5 mRNA is regulated following amphetamine administration. We examined RGS5 knock-out mice in several paradigms to evaluate locomotor activity, motor coordination and learning, anxiety–like behavior and behavioral responses to psychostimulants (Ena et al., in preparation).

Altogether, we developed a reliable method applied on adult brain to identify and generate specific striatopallidal and striatonigral neuron gene profiles. Our approach led to the identification of new striatopallidal and striatonigral neuron-specific genes. Finally, our results highlighted the central role of an ecto-nucleotidase associated to adenosine receptors in striatal-dependent learning.

As a perspective, we have selected a series of six genes that are selectively expressed in one MSN subpopulation and that will be now characterized in terms of their functional involvement in the basal ganglia system and its pathologies by using conditional gene inactivation.

1.5 Expression and functional roles of SV2C in striatal and mesencephalic neuronal subpopulations

Synaptic vesicle 2 proteins (SV2), SV2A, SV2B and SV2C, are integral proteins localized on the surface of synaptic vesicles in all neurons. SV2 proteins appear to play an important, but not yet fully understood role in synaptic vesicle exocytosis and neurotransmitter release. Moreover, SV2 seems to be the receptor of the botulinum neurotoxin A. Using single and double-labeling fluorescent immunohistochemistry and *in situ* hybridization, we have identified the brain pattern of SV2C mRNA and protein expression in mice. Our results indicated that SV2C protein was expressed in a small subset of brain regions including olfactory bulb, olfactory tubercle, nucleus accumbens, caudate-putamen, ventral pallidum, globus pallidus, substantia nigra and ventral tegmental area (Dardou et al., 2011). These results were confirmed by *in situ* hybridization, except for the globus pallidus and the substantia nigra pars reticulata, in which no labeling was found, suggesting that SV2C positive-fibers in these areas are terminals of striatal MSN projecting neurons. In the striatum, we found that, in addition to its presence in the projection neurons, SV2C was densely expressed in a fraction (around 45%) of cholinergic interneurons. In addition, our data also showed that SV2C was densely expressed in most dopaminergic neurons in substantia nigra pars compacta and ventral tegmental area (more than 70% of the dopaminergic neurons analyzed were SV2C-positive) (Dardou et al., 2011).

We also investigated the implication of SV2C in both normal and pathological basal ganglia functioning by using SV2C-deficient or SV2C-knock down mice. We showed that in SV2C-deficient mice ($SV2C^{-/-}$)

mice, the expression of tyrosine hydroxylase mRNA in midbrain dopaminergic neurons was largely and significantly increased whilst enkephalin mRNA expression was significantly decreased in the caudate-putamen and accumbens nucleus (Dardou et al., 2013). In two models of dopaminergic denervation, SV2C mRNA expression was significantly increased in the striatum. In order to further understand the role of SV2C, we performed behavioural experiments on SV2C$^{-/-}$ mice and on knock-down mice receiving an injection of adeno-associated virus expressing SV2C miRNA specifically in the ventral midbrain. We showed that even if these modifications of SV2C expression had no impact on behaviour in open field and elevated plus maze, the specific knock-down of SV2C expression in the dopaminergic neurons completely abolished the development of a conditioned place preference induced by cocaine while the reaction to an acute drug injection remains similar in these mice compared to control mice (Dardou et al., 2013). These results suggest that SV2C is involved in normal operation of the basal ganglia network and could also be involved in system adaptation in basal ganglia pathological conditions.

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

Striatal neurons (MSN) excitability and corticostriatal synaptic transmission are tightly regulated both by a large series of neurotransmitters and by striatal interneurons.
Among the four classes of interneurons, striatal fast spiking interneurons (FSI) modulate the output of the striatum by providing a powerful feedforward inhibition on striatal MSN and synchronizing their activity. Recent studies have broadened our understanding of FSI, showing that they are implicated in severe motor disorders as Parkinsonism, dystonia and Tourette syndrome. FSI are the only striatal neurons to express the Ca^{2+}-binding protein parvalbumin (PV). This selective expression of PV raises questions about the functional role of this Ca^{2+} buffer in controlling FSI Ca^{2+} dynamics, and, consequently, the FSI spiking mode and neurotransmission. Therefore, to study the functional involvement of FSI in striatal microcircuit activity and the role of PV in FSI function, we performed perforated patch recordings on EGFP-expressing FSI in brain slices from control and PV-/- mice (Orduz et al., 2013). Our results revealed that PV-/- FSI fired more regularly and were more excitable than control FSI by a mechanism in which Ca^{2+} buffering seems to be linked to spiking activity as the result of the activation of small conductance (SK) Ca^{2+}-dependent K^+ channels (Orduz et al., 2013). Numerical simulations in a mathematical model could be a suitable approach to verify this hypothesis. However, so far the existing conductance-based computational models for FSI did not allow the study of the coupling between PV concentration and electrical activity. Therefore, we generated a new mathematical model for the FSI that includes apamin-sensitive SK channels and takes into account the presence of a Ca^{2+} buffer (Bischop et al., 2012). We found that this modeling approach of striatal FSI fully supported our experimental results by showing, for instance, that a variation in the concentration of PV substantially modulates the intrinsic excitability of FSI (Bischop et al., 2012; Orduz et al., 2013).

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

between 20 to 50 ms, they exhibit a paired-pulse depression in wild-type that is reversed to a clear paired-pulse facilitation in PV-/- mice (Orduz et al., 2013).
Altogether, our results demonstrated that in FSI, PV is crucial for the fine-tuning of the temporal responses of the FSI network and for the orchestration of MSN populations. This, in turn, may play a direct role in the generation and pathological worsening of motor rhythms.

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

- The transgenic A_{2A}R-Cre mouse strain that we previously developed (Durieux et al., 2009,2011) was also used in a collaborative study to better understand the functions of A_{2A}Rs receptors in physiological processes by mapping projections of A_{2A}R-expressing neurons utilizing adeno-associated virus (AAV) encoding humanized Renilla green fluorescent protein (hrGFP) as a tracer for long axonal pathways. The Cre-dependent AAV was injected into the core (AcbC) and shell (AcbSh) of the nucleus accumbens (Acb) in A_{2A}R-Cre mice. Immunohistochemistry was then used to visualize hrGFP, highlighting the perikarya of the A_{2A}R neurons in the injection sites, and their axons in projection regions. The data revealed that A_{2A}R neurons exhibit round, middle-sized, and elliptic perikarya with their processes within the AcbC and AcbSh. Moreover, the projections from the Acb distributed to nuclei in the forebrain, diencephalon, and brainstem. In the forebrain, A_{2A}R neurons jointly projected to the ventral pallidum, the nucleus of the diagonal band, and the substantia innominata. Heavy projections from the AcbC and the ventral AcbSh, and weaker projections from the medial AcbSh, were observed in the lateral hypothalamus and lateral preoptic area. In brainstem, Acb projections were found in the ventral tegmental area, while AcbC and ventral AcbSh also projected to the medial raphe nucleus, dorsal raphe nucleus, and the ventrolateral periaqueductal gray.

- The contribution of neuronal dysfunction to neurodegeneration was studied in a transgenic mouse model of spinocerebellar ataxia type 1 (SCA1) displaying impaired motor performance ahead of loss or atrophy of cerebellar Purkinje cells. (Hourez et al., 2011). We first showed that presymptomatic SCA1 mice exhibit a reduction in the firing rate of Purkinje cells (both in vivo and in slices) associated with a reduction in the efficiency of the main glutamatergic synapse onto Purkinje cells and with an increase in A-type K+ current (IK_A). This early functional alteration in Purkinje cells causes motor dysfunction before the appearance of any atrophy or neuronal death. Moreover, we also demonstrated in this SCA1 model that neuronal dysfunction contributes to neurodegeneration of cerebellar Purkinje cells. Indeed, our results 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). This chronic treatment with 3.4-diaminopyridine is associated with increased cerebellar levels of BDNF suggesting that partial protection against atrophy of Purkinje cells is possibly provided by an increased production of growth factors secondary to the re-increase in electrical activity. 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.

- In collaboration with the group of Martin Schwab from the Brain Research Institute at University of Zurich, we investigated the function of neuronal Nogo-A. Nogo-A is a membrane protein enriched in the adult central myelin, where it restricts the capacity of axons to grow and regenerate after injury

but is also expressed by certain neurons, in particular during development, where its physiological function was less well understood. In the cerebellum, Nogo-A is transitorily highly expressed in the Purkinje cells (PC) during early postnatal development. By using Nogo-A-deficient (Nogo-$A^{-/-}$) mice and mice with a selective overexpression of Nogo-A in PC and patch clamp recordings as well as 3D confocal reconstruction of recorded PC, we analyzed its effect on dendritogenesis and on the formation of their main input synapses from parallel (PF) and climbing fibers (CF) (Petrinovic et al., 2013). PC dendritic trees were larger and more complex in Nogo-$A^{-/-}$ mice and smaller than in wild-type in Nogo-A overexpressing PC. Nogo-$A^{-/-}$ resulted in premature soma-to-dendrite translocation of CF and an enlargement of the CF territory in the molecular layer during development. Although spine density was not influenced by Nogo-A, the size of postsynaptic densities of PF-PC synapses was negatively correlated with the Nogo-A expression level. Moreover, PC patch clamp recordings revealed that Nogo-A negatively regulates the strength of synaptic transmission at the PF-PC synapse. Thus, our results demonstrated that Nogo-A is a negative regulator of PC input synapses which orchestrates cerebellar connectivity through regulation of synapse morphology and size of the PC dendritic tree (Petrinovic et al., 2013). They identified Nogo-A as a putative negative controller of synaptic development, strength and structure in several brain regions.

- We took part to the study of Jens Eilers and collaborators from the Carl-Ludwig Institute for Physiology at University of Leipzig on the coupling between presynaptic Ca^{2+} and transmitter release and hence synaptic transmission (Schmidt et al., 2013). The coupling distance between presynaptic Ca^{2+} influx and the sensor for vesicular transmitter release determines speed and reliability of synaptic transmission. We have analyzed excitatory PF to PC synapses in the cerebellum of wild-type mice and mice deficient in the Ca^{2+} binding protein calretinin, specifically expressed in the granule cell and their axons (PF) (Schiffmann et al., 1999). The coupling distance was quantified by combining fluctuation analyses in patch clamp recordings, presynaptic Ca^{2+} imaging, and reaction-diffusion computer simulations. We found a coupling distance of <30 nm at these synapses, much shorter than at any other glutamatergic cortical synapse investigated to date. Our results suggest that nanodomain coupling is a general characteristic of conventional cortical synapses involved in high-frequency transmission.

- We took part to the functional characterization of human embryonic (ESC) and induced pluripotent (iPSC) stem cells differentiated in functional pyramidal neurons (Espuny-Camacho et al., 2013) that have been generated and characterized by the Pierre Vanderhaeghen's lab. By first using calcium imaging, we demonstrated spontaneous calcium waves that were blocked by tetrodotoxin. We next demonstrated by performing patch-clamp recordings a clear temporal evolution in the electrophysiological properties of the cell population, with increased proportion of cells displaying spontaneous synaptic currents (EPSCs) and repetitive firing (Espuny-Camacho et al., 2013). This was corroborated at the single cell level by the observation of an increase with time in the intensity of the voltage dependent sodium current and the amplitude of action potentials. Together with other in vitro and in vivo data, these results demonstrate that human cortical neurons generated *in vitro* from ESC/iPSC can develop complex hodological properties characteristic of the cerebral cortex *in vivo*.

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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

1. Introduction to the cystine/glutamate antiporter (system x_c^-) & its functions in the brain

The cystine/glutamate antiporter or system x_c^- is a membrane-bound Na^+-independent amino acid transporter that is structurally composed of a heavy chain subunit common to all amino acid transporters, 4F2, and a light chain specific subunit, xCT (Sato et al, J Biol Chem 1999; 274:11455-11458). Disturbances in functioning of brain system x_c^- can have dual physiological implications. Indeed, this antiporter provides cells with cystine that is intracellularly reduced to cysteine, the rate-limiting building block of the major brain antioxidant glutathione (GSH) and activation of system x_c^- can thus protect the brain against oxidative stress.

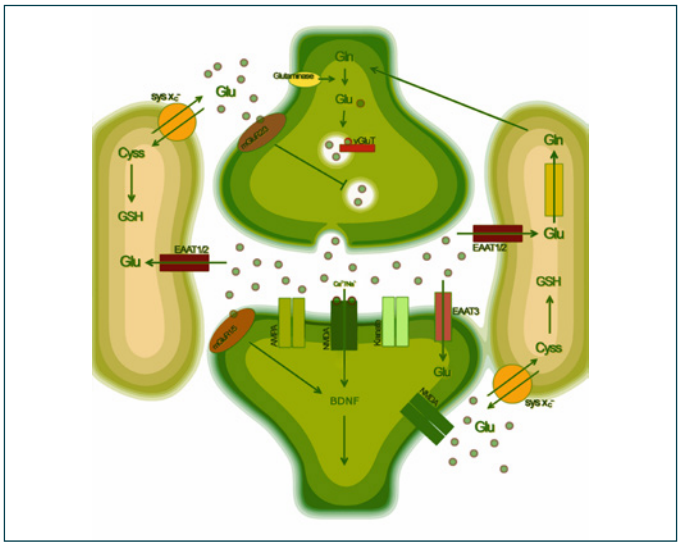


Figure 1 Key proteins of glutamate transmission

However, system x_c^- is also a source of non-vesicular glutamate, meaning that activation of system xc- in response to oxidative stress, can be a trigger to induce excitotoxic cell death.

System x_c^- has received considerable attention because it is upregulated in various cancers, including malignant brain glioma (Chung et al, J Neurosci 2005, 25:7101-7110). A large body of evidence indicated that disruptions in glutamate homeostasis and neuroadaptations in system x_c^- are associated with addictive behaviours to drugs of abuse and that targeting system x_c^- inhibits drug-seeking and relapse in rodents and humans (Kalivas, Nat Rev Neurosci 2009; 284:1106-1115).

With regard to neurological disorders, most of the work has focussed on *in vitro* studies, from which it became clear that system x_c^- has a pivotal function in supporting GSH biosynthesis for survival and proliferation of many cell types (Albrecht et al, CNS Neurol Disord Drug Targets 2010; 9:373-382). However, *in vivo* studies investigating to which extent dysfunction of system x_c^- affects GSH levels and oxidative stress in the brain were lacking at the time we initiated this project and became thus the subject of our interest.

We first investigated whether homozygous deletion of the specific system x_c^- subunit xCT ($xCT^{-/-}$ mice) affected brain GSH content and oxidative stress-related markers before looking further into animal models for neurological disorders. In a first study, we focussed on the nigrostriatal pathway and a mouse model for Parkinson's disease, known to be dependent on oxidative stress-related neuronal

damage. xCT deletion did not affect striatal GSH levels, and no signs of increased oxidative stress were observed in striatum or substantia nigra of xCT$^{-/-}$ mice under physiological conditions. We did notice a decrease of 70% in striatal extracellular glutamate levels, and xCT$^{-/-}$ mice were clearly less susceptible to 6-hydroxy-dopamine-induced neurodegeneration in the substantia nigra compared to age-matched wild-types (Massie et al., FASEB J 2011;25:1359-1369).

2. Hypothesis

The above described novel findings thus sustained that system x_c^- is the major source of extracellular glutamate in the striatum, and that genetic deletion of xCT is neuroprotective in a hemi-Parkinson disease model. This breakthrough was the fundament to further unveil the role and mechanisms of action of system x_c^- in other pathophysiological conditions, e.g. temporal lobe epilepsy (TLE) and comorbid depressive symptoms and cognitive decline.

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

The innovative nature of the current proposal is twofold. First, it defines a completely new drug target for epilepsy, and second, this target is localised on glial cells and not on adult nerve cells. 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 a relevant approach that can have a major impact on the field of epilepsy.

3. Results obtained between 2011- 2013

3.1 Summary of the manuscript published in the Journal of Neuroscience 31 (2011) 5792-5803; Loss of system xc- does not induce oxidative stress but decreases extracellular glutamate in hippocampus and influences spatial working memory and limbic seizure susceptibility (SCI impact factor = 7.115)

We unravelled the importance of system x_c^- in hippocampal functioning in both baseline and disease conditions. Deletion of xCT had no effect on hippocampal GSH content or oxidative stress markers. With ageing xCT$^{-/-}$ mice did not display exacerbated cortical thinning, hippocampal atrophy or glial cell loss in the hippocampus. xCT$^{-/-}$ mice learned both the procedural and spatial aspects of the water maze task and displayed intact spatial reference memory. Together these results indicate that loss of system x_c^- does not induce oxidative stress *in vivo*. Yet, young xCT$^{-/-}$ mice displayed a deficit in the continuous Y-maze spontaneous alternation task, indicating partial spatial working memory impairment.

We next observed significantly lower extracellular hippocampal glutamate levels in xCT$^{-/-}$ mice compared to wild type littermates. Moreover, intrahippocampal perfusion with system x_c^- inhibitors lowered extracellular glutamate whereas the system x_c^- activator N-acetylcysteine elevated hippocampal glutamate levels. 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 acute limbic seizures evoked by pilocarpine, NMDA or kainic acid.

Moreover N-acetylcysteine had proconvulsive effects in the kainic acid and the 6Hz corneal stimulation model. In the former model, these proconvulsive effects were abolished in xCT$^{-/-}$ mice

Noteworthy, a microarray screening performed on hippocampal mRNA samples of xCT$^{-/-}$ mice and wild type littermates, in order to identify possible compensatory changes in the xCT$^{-/-}$ mice as a result of the genetic knockout of xCT, did not report changes in genes related to oxidative stress or glutamate excitotoxicity (unpublished data).

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 (De Bundel et al. J Neurosci 2011;31:5792-5803).

3.2 Our contribution to the manuscript published in Antioxidants and Redox Signalling (Epub ahead of print); Phosphoinositide 3-kinases upregulate system x_c^- via eIF2α and ATF4 – a pathway active in glioblastomas and epilepsy (SCI impact factor 2012 = 7.189)

Phosphoinositide 3-kinases (PI3K) relay growth factor signalling and mediate cytoprotection and cell growth. As described in the introduction, system x_c^- imports cystine while exporting glutamate, thereby promoting GSH synthesis while increasing extracellular cerebral glutamate. The aim of this study was to analyse the pathway through which growth factor and PI3K signalling induce the cystine/glutamate antiporter system x_c^- and to possibly demonstrate its biological significance for epilepsy.

PI3Ks induce system x_c^- through glycogen synthase kinase 3β (GSK-3β) inhibition, general control non-derepressible-2 (GCN2)-mediated phosphorylation of the translation initiation factor eIF2α (phosphor-eIF2 α) and subsequent translational upregulation of the transcription factor ATF4 (Figure 2). This pathway is essential for PI3Ks to modulate oxidative stress resistance of nerve cells and insulin-induced growth in fibroblasts.

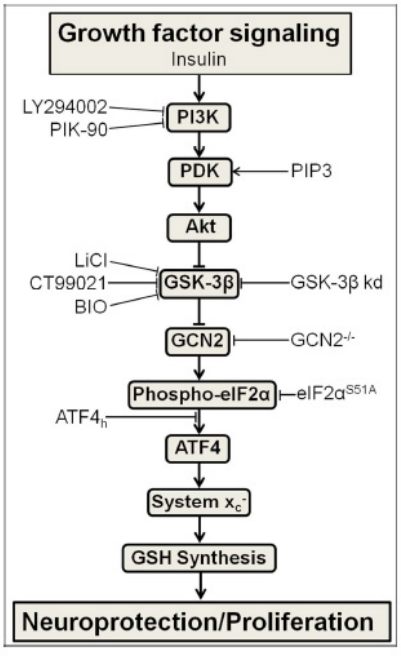


Figure 2 – The PI3K/GSK3β/eIF2α/ATF4/xCT signalling pathway

This new signalling pathway was induced in primary cortical neurons in response to robust neuronal activity and was also upregulated in the human hippocampal tissue samples of TLE patients. Indeed, Western blotting performed on five samples of hippocampal tissue from patients with intractable TLE treated with temporal lobectomy compared to five control samples revealed a strong increase in Akt and GSK3β phosphorylation in the epileptic tissue which was associated with a significant increase in eIF2α phosphorylation, ATF4 and xCT protein expression (Figure 3).

To support the view that the observed differences between the groups actually represent the activity of the PI3K/GSK3β/eIF2α/ATF4/xCT pathway in the human brain, we performed a linear regression analysis testing the pairwise relationship of the four pairs of connected parts of the pathway across the whole group of samples (Figure 3). This showed positive results for all pairs. Moreover, the linear association of GSK3β and eIF2α phosphorylation showed a goodness of fit (r^2=0.72) comparable to those of well-established signalling modules Akt and GSK phosphorylation (r^2=0.77) and eIF2α phosphorylation and ATF4 protein expression (r^2=0.69).

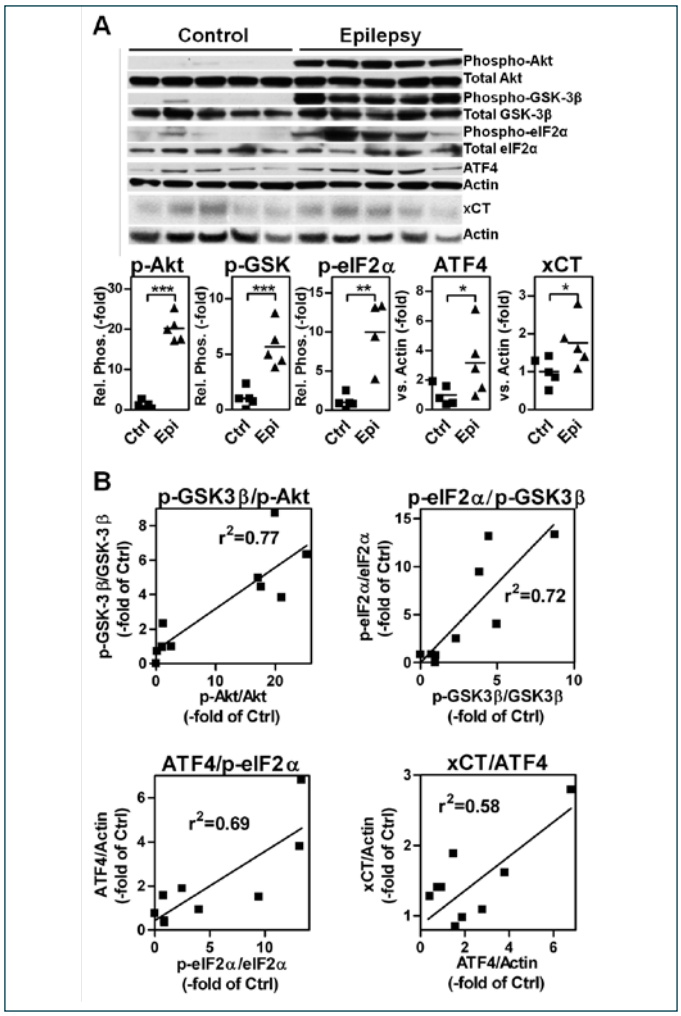


Figure 3 - The PI3K/GSK3β/eIF2α/ATF4/xCT pathway is activated in human epileptic hippocampal tissue.
Protein extracts from surgical specimens from human epileptic hippocampi (Epilepsy) and control hippocampi (Control) were tested for the relative phosphorylation of Akt (phospho-Akt), GSK3β (phospho-GSK3β)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

In conclusion, our findings further extend the concepts of how growth factors and PI3Ks induce neuroprotection and cell growth by adding a new branch to the signalling network downstream of GSK3β, which ultimately leads to the induction of the cystine/glutamate antiporter system x_c^-. Importantly, induction of this pathway by neuronal activity and in epileptic hippocampi points to a potential role in the pathophysiology of non-tumor-associated epilepsy by upregulating extracellular cerebral glutamate.

3.3 Summary of the manuscript submitted to Annals of Neurology; Lack of effect of zonisamide treatment on glutathione (GSH) levels and system x_c^-.

Recently, Asanuma and coworkers (Asanuma et al., Ann Neurol 2010;67:239–249) reported that systemic zonisamide (ZNS) treatment increases GSH levels in mouse basal ganglia as well as in astroglial C6 cells and this increase was attributed to enhancement of system x_c^-. However, in our lab we observed that loss of system x_c^- is neuroprotective in a mouse model for Parkinson's disease (PD) and we linked this observation to the decreased extracellular glutamate levels that are observed in the striatum of system x_c^--deficient mice (Massie et al., FASEB J 2011;25:1359-1369). Our hypothesis that inhibition of system x_c^- might result in neuroprotection in PD thus contradicts the recent report by Asanuma and co-workers who attributed neuroprotective effects of ZNS to the enhancement of system x_c^- and subsequent increase in GSH content. Since both the mechanism(s) of action of the neuroprotective effects of ZNS as well as the role of system x_c^- in various neurological disorders are hot topics in neuroscience research nowadays, we investigated this discrepancy between both our hypotheses.

With our study we aimed at repeating the experiments described by Asanuma and co-workers, in $xCT^{-/-}$ mice and their wildtype littermates ($xCT^{+/+}$). As such, we wanted to investigate whether the increase in GSH after ZNS treatment was dependent on system x_c^-. By coincidence we found out that, while we were performing these in vivo experiments, one of our collaborators was doing similar experiments with ZNS in vitro. Finally, on our demand, a third independent lab repeated some additional in vitro experiments to investigate whether ZNS treatment increases system x_c^- activity and/or intracellular GSH content.

In short, after 30 mg/kg ZNS (i.p.) treatment for two weeks we could not observe any change in GSH levels in basal ganglia of $xCT^{+/+}$ nor $xCT^{-/-}$ mice (Figure 4 A,B). Moreover, we could not observe any difference in xCT expression level in the striatum or ventral midbrain of the $xCT^{+/+}$ mice (Figure 4 C,D). These data are in sharp contrast to the observations of Asanuma and co-workers. Since our goal was to reproduce the effect of ZNS on GSH levels and to compare it in $xCT^{-/-}$ and $xCT^{+/+}$ mice, we have no idea about a possible reason for the discrepancy between our study and the study of Asanuma and co-workers. Besides the genetic background of the mice, environmental factors could influence the data.

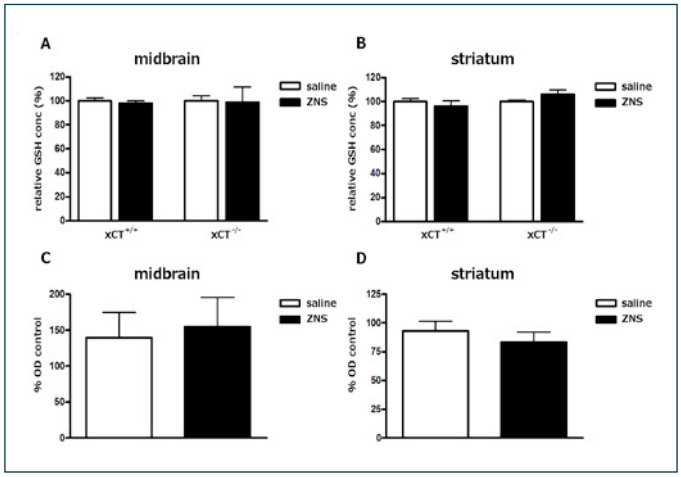


Figure 4. Effect of a 14 day zonisamide (ZNS) treatment on (A) midbrain and (B) striatal glutathione (GSH) levels in $xCT^{-/-}$ mice and their $xCT^{+/+}$ littermate. Bars represent mean±SEM (midbrain: $xCT^{-/-}$ p=0.286 (n=5), $xCT^{+/+}$ p=0.686 (n=4); striatum: $xCT^{-/-}$ p=0.222 (n=5), $xCT^{+/+}$ p=0.730 (n=5)). Quantification of (C) midbrain and (D) striatal xCT expression levels by Western blotting in $xCT^{+/+}$ mice after a two week treatment with ZNS compared to saline controls. Bars represent mean±SEM (midbrain: p=0.686 (saline n=5, ZNS n=4); striatum: p=0.413 (saline n=5, ZNS n=4)).

However, also our in *vitro data* do not support the hypothesis that ZNS might enhance system x_c^- and as such exert its neuroprotective effect (Figure 5).

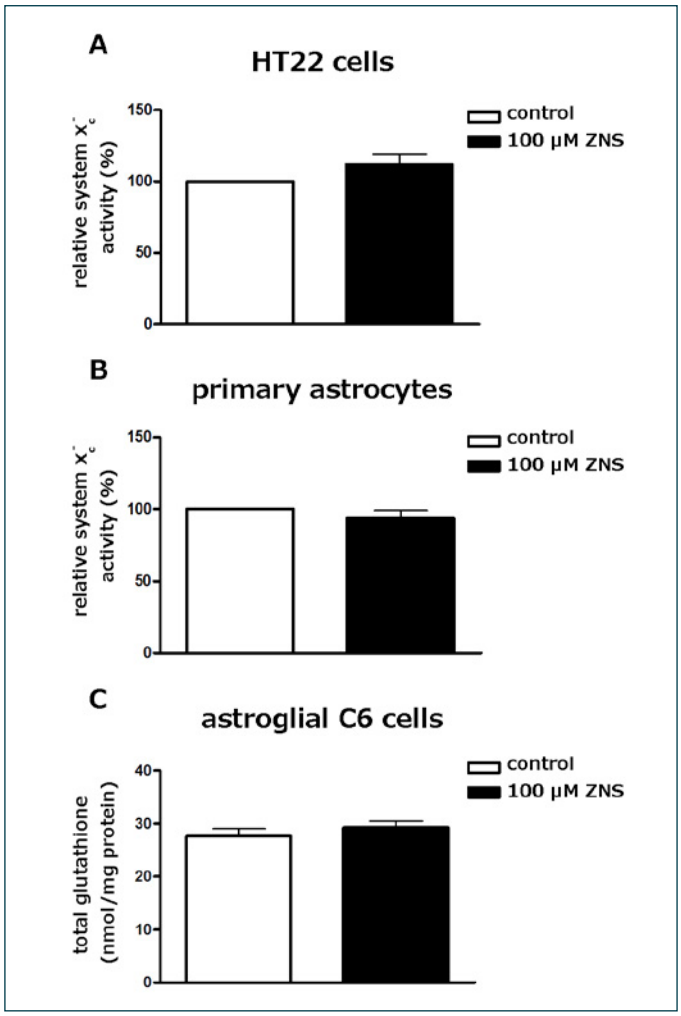


Figure 5. Effect of 100 µM zonisamide (ZNS) administration on relative system x_c^- activity in (A) HT22 cells upon 24h of ZNS and (B) microglia-free murine primary astrocytes upon 48h of ZNS and (C) on intracellular glutathione content in astroglial C6 cells upon 24h of ZNS treatment. The graphs show the mean±SEM of six (A) and five (B, C) independent experiments (HT22 cells: p=0.132; primary astrocytes: p=0.316; astroglial C6 cells: p=0.548).

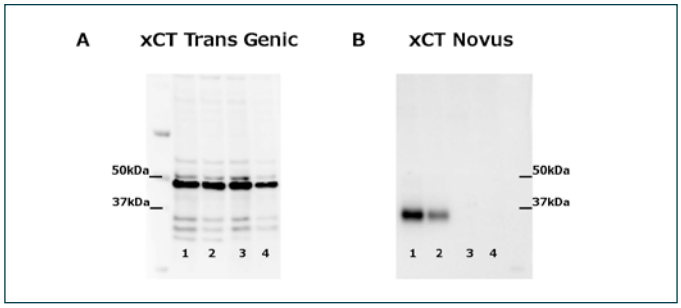


Figure 6. A representative example of a Western blotting experiment (2 concentrations of each sample were loaded; lane 1-2: $xCT^{+/+}$ mouse brain tissue samples; lane 3-4: $xCT^{-/-}$ mouse brain tissue samples). (A) No specific bands are observed after incubation with the xCT Trans Genic Inc. antibody (KE021). (B) After incubation with the xCT Novus Biologicals antibody (NB300-318) specific xCT bands are observed ~35 kDa in the $xCT^{+/+}$ samples which are absent in the $xCT^{-/-}$ samples.

Importantly, in this study we draw attention to a major problem that is encountered in xCT research nowadays, resulting from a broad supply of commercial xCT antibodies (see also III.5). The data on expression of xCT after ZNS treatment were collected using an antibody that, at least in our hands, does not specifically label xCT by Western Blotting. The use of $xCT^{-/-}$ tissue as a negative control is mandatory to correctly identify xCT. Otherwise, unspecific signals can easily be considered as xCT protein, resulting in misinterpretation of the data. Since we could not obtain a specific signal with the xCT antibody (1/100; Trans Genic Inc., KE021) that was used by Asanuma and co-workers (Figure 6A), we used a different rabbit antibody raised against xCT (1:5,000; Novus Biologicals, NB300-318) (Figure 6B). The Trans Genic Inc. xCT antibody labels a protein of ~50 kDa, a molecular weight that is often attributed to xCT (Kim et al., Biochim Biophys Acta 2001;1512:335-344). However, this band can be seen in $xCT^{-/-}$ brain tissue homogenates. Moreover, as shown in fig. 6B and in Massie et al., 2008 (Neuroreport 2008;19:1589-1592), the specific xCT band is located at ~35kDa.

In conclusion, our data do not exclude that ZNS induces increased GSH levels under pathological conditions, which were not subject of our study. Despite our negative results, there is a large body of evidence that ZNS is a promising neuroprotective drug. However, the data of our study strongly suggest that its neuroprotective effects are mediated by a system x_c^-- independent mechanism.

3.4. New recent data - Levetiracetam does not increase GSH levels nor system x_c^- expression in mouse hippocampus

We also administered levetiracetam (LEV) to other groups of $xCT^{-/-}$ and $xCT^{+/+}$ mice to test the hypothesis of Ueda and coworkers (Ueda et al., Brain Res 2007;1151:55-61). Their findings suggest that LEV exerts a neuroprotective role, possibly in part by modifying xCT and iNOS expression levels and affecting the activity of the cysteine/glutamate antiporter and subsequent endogenous antioxidant abilily. This last suggestion thus implies increased activity of system x_c^- and subsequent increase in GSH content which contradicts our hypothesis that inhibition of system x_c^- might result in neuroprotection. Recently, we observed that loss of system x_c^- decreased extracellular hippocampal glutamate levels without affecting hippocampal GSH levels (De Bundel et al. J Neurosci 2011;31:5792-803).
After two weeks of LEV (54 mg/kg i.p.) treatment we could not observe any change in GSH levels in hippocampus of $xCT^{+/+}$ nor $xCT^{-/-}$ mice. In addition, xCT expression level in hippocampus of the $xCT^{+/+}$ mice were unaltered after LEV administration (Figure 7).

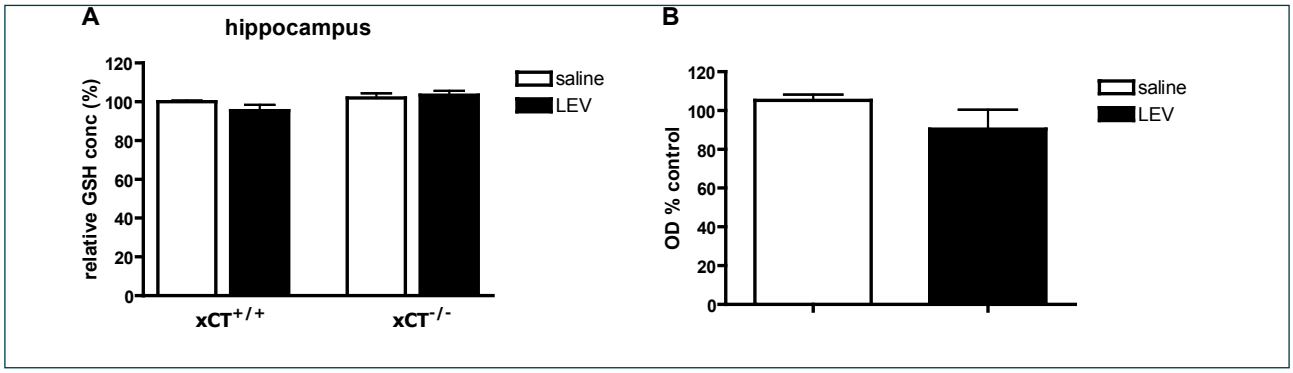


Figure 7. (A) Effect of a 14 day levetiracetam (LEV) treatment on hippocampal glutathione (GSH) levels in $xCT^{-/-}$ mice and their $xCT^{+/+}$ littermate. Bars represent mean±SEM ($xCT^{-/-}$ p=0.886 (n=5), $xCT^{+/+}$ p= 0.343 (n=5)). (B) Quantification of hippocampal xCT expression levels by Western blotting in $xCT^{+/+}$ mice after a two week treatment with LEV compared to saline controls. Bars represent mean±SEM (p=0.556 (saline n=5, LEV n=5)).

These data are in contrast to the findings of Ueda and co-workers. In this case the discrepancy between our study and the study of Ueda and co-workers might be explained by the fact that the original experiments were conducted on rats and we administered the same dose of LEV to our mice. However, in this study we encountered the same problem as described before, Ueda and coworkers used the Trans Genic Inc. xCT antibody to determine xCT expression. The authors displayed the result of an xCT blot with the Trans Genic Inc. xCT antibody which labels a protein of ~50 kDa, similar to our own blot shown above.

In conclusion, our data show that systemic treatment of mice with 54 mg/kg LEV for two weeks does not alter hippocampal GSH levels or xCT expression levels. In order to strongly state that the neuroprotective effects of LEV are mediated by a system x_c^-- independent mechanism hippocampal iNOS expression levels could be determined and a dose-response study should be conducted in future experiments.

3.5 New recent data - Development of new x_c- research tools: In search for an xCT antibody that can be used for immunohistochemistry Distribution of system x_c- in mouse brain

As described above, a major problem in xCT research nowadays is the use of commercially available or home-made xCT antibodies without checking whether or not xCT is specifically labeled. The use of $xCT^{-/-}$ tissue as a negative control is mandatory to correctly identify xCT. Otherwise, unspecific signals can easily be considered as xCT resulting in misinterpretation of the data.
Therefore, we have been screening a batch of 40 new antibodies (obtained from Dr. N.C. Danbolt, Oslo, Norway) and several commercial xCT antibodies by Western Blotting. The antibodies that displayed a specific xCT band, i.e. located at ~35kDa and absent in $xCT^{-/-}$ tissue, were subsequently tested in immunohistochemistry staining protocols with distinct tissue pretreatment:
*perfusion 4% paraformaldehyde (PF) + vibratome slices 40µm
*perfusion 1% PF + vibratome slices 40µm
*perfusion 4%PF + sucrose protection + cryosections 20µm
*perfusion (4%PF + 0.35%glutaraldehyde) + sucrose protection + cryosections 20µm
*snap freeze + cryosections 20µm + postfixation: 4%PF
*snap freeze + cryosections 20µm + postfixation: ethanol
***snap freeze + cryosections 20µm + postfixation: aceton**
Only the last pretreatment protocol (here in bold) leads to specific di-aminobenzidine (DAB) staining of xCT on $xCT^{+/+}$ brain slices which is absent on $xCT^{-/-}$ slices (Figure 8).

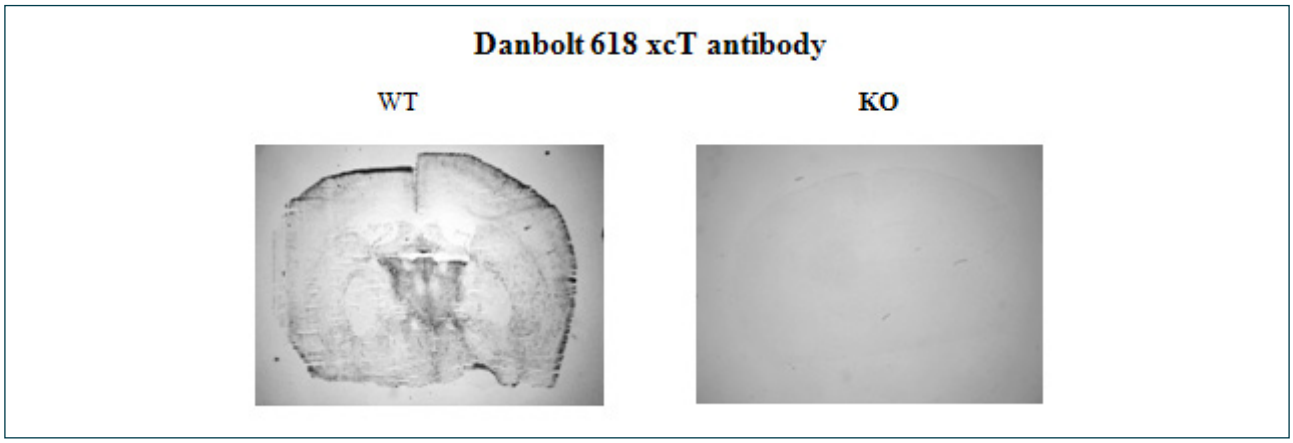


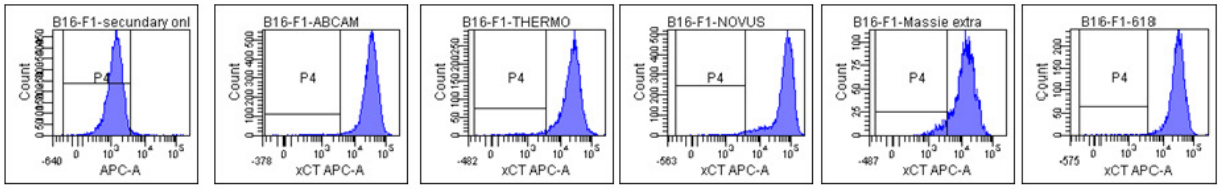
Figure 8. A representative example of an immunohistochemical staining experiment in which $xCT^{+/+}$ and $xCT^{-/-}$ cryosections (20µm), post fixated with aceton, are stained with the Danbolt 618 xCT antibody.

However, Figure 8 also clearly demonstrates that it will be impossible to determine the cellular distribution of system x_c^- using immunohistochemistry due to the poor quality of the cryosections with aceton postfixation. Based on these stainings we will only be able to determine the relative distribution of system x_c^- in major brain regions.

Consequently, we are currently optimizing an extraction protocol to obtain a single cell suspension of $xCT^{+/+}$ and $xCT^{-/-}$ mouse brain cells, in which we will detect xCT using flow cytometry. In short, the cell suspension will be divided into multiple samples and labeled with distinct xCT antibodies in combination with markers for distinct brain cells, i.e. neurons, astrocytes, microglia and endothelial cells. The suitability for flow cytometry and specificity of the home-made and commercial antibodies has already been determined on tumor cells and on splenocytes (Figure 9).

This particular technical realization that we are currently finalizing, will be of utmost importance for further research in neuroscience on system x_c^- (Van Liefferinge et al., in preparation).

A Tumor cell line B16



B Splenocytes

mature T lymphocytes (CD3)

myeloid-derived suppressor cells (CD11b+GR1)

macrophages (CD11b+F4/80)

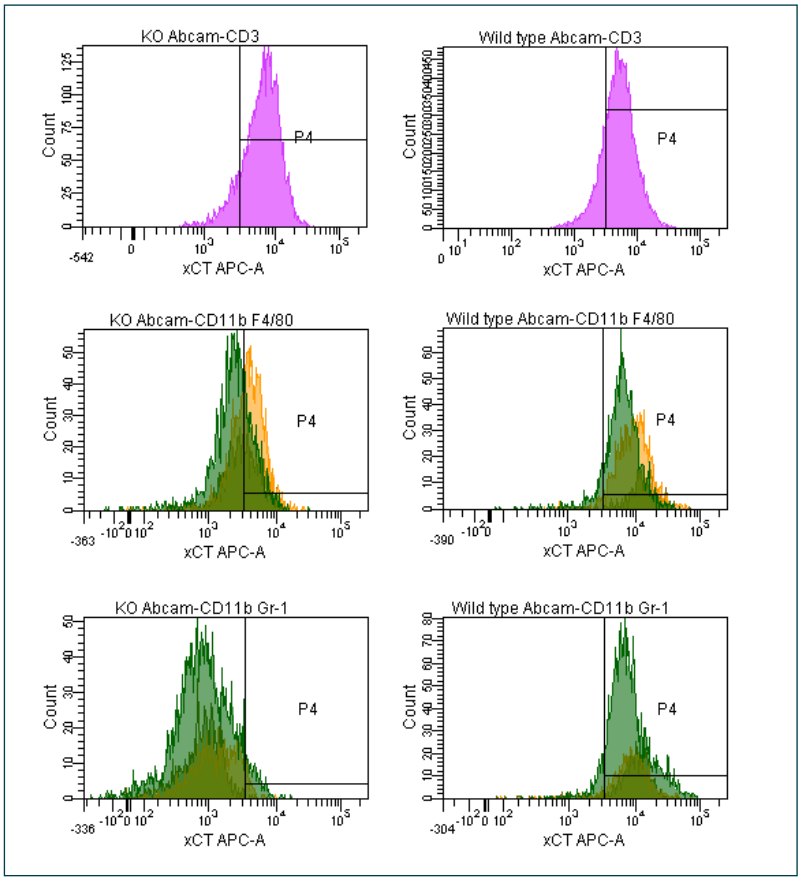


*Figure 9. Representative examples of a flow cytometric experiments on **(A)** tumor cells and **(B)** splenoctyes. **(A)** Tumor cells were labeled with distinct xCT antibodies to determine the suitability of the antibodies for flow cytometry. A clear shift to the right is observed for all depicted xCT antibodies, which implies they all display a higher fluorescent intensity, distinct from the aspecific signal of the secondary fluorescent antibody. **(B)** Splenocytes of both $xCT^{+/+}$ and $xCT^{-/-}$ were labeled with xCT Abcam antibody (ab37185) to determine the specificity of this antibody. Mature T-lymphocytes do not express system x_c^-, while MDSC cells and macrophages clearly display a shift to higher fluorescent signaling in xCT wildtype spleen tissue compared to $xCT^{-/-}$ tissue. With this experiment we confirmed the data of Srivastava et al. (Cancer Res 2010;70:68-77).*

3.6 New recent data - Role of system x_c^- in refractory epilepsy and epileptogenesis

The chronic mouse models we are currently using to investigate and mimic TLE in mice are the amygdala kindling model, the post-status epilepticus (SE) pilocarpine model and the 6Hz corneal kindling model. The first two models are well-accepted models for epileptogenesis, reliably inducing a persistent epileptic state, demonstrable by behavioral and/or EEG assessment. The 6Hz corneal kindling model on the other hand is a recently validated model for refractory epilepsy.

We are currently studying xCT mRNA and protein expression levels and xCT distribution patterns during the various phases of the chronic pilocarpine model for TLE, using NMRI mice. This post-SE model was conducted as described by Mazzuferi et al. 2012 (Mazzuferi et al. Exp Neurol 2012;238:156-167). SE was induced by i.p. administration of pilocarpine and stopped after 3 hours by a diazepam i.p. injection. During the experiments behavioral and EEG data were collected by 24h video-EEG monitoring.

We observed a significant change in xCT expression at 4 weeks after pilocarpine-induced SE (Figure 10).

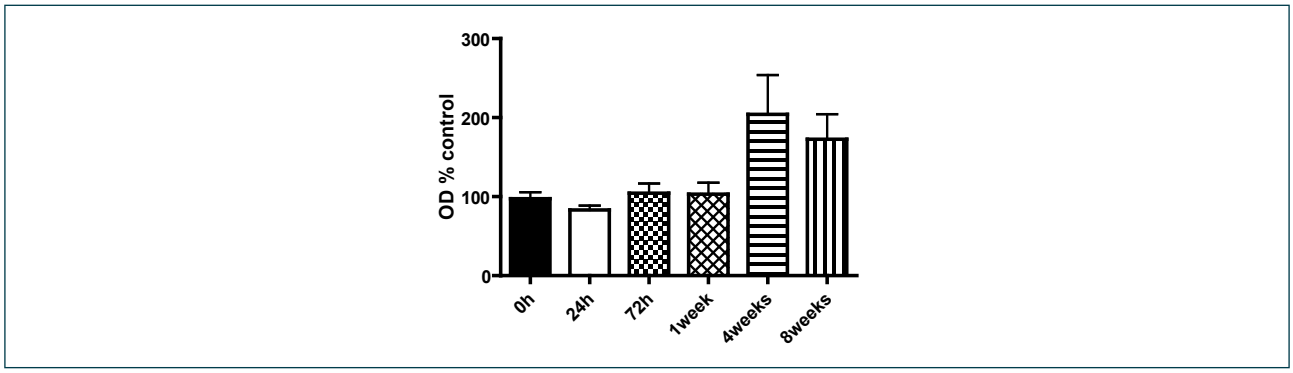


Figure 10. Quantification of hippocampal xCT expression levels by Western blotting in NMRI mice at 0h, 24h, 72h, 1 week, 4 weeks and 8 weeks after status epilepticus (SE) induced by i.p. administration of pilocarpine. Optical densities were measured relative to a pool sample and normalized to a total protein stain. Bars represent mean±SEM (n=6; one way ANOVA with Dunnett's Multiple Comparison Test, p= 0.045).

$xCT^{-/-}$ and $xCT^{+/+}$ mice (C57BL/6 background) are also subjected to the various clinically relevant chronic mouse models and we will compare whether deletion of xCT will affect the process and/or severity of epileptogenesis.

At the moment we are running a final batch of $xCT^{-/-}$ and wildtype mice in the 6Hz corneal kindling model. Preliminary results of this model are shown in Figure 11. In this model the process of epileptogenesis is mimicked by twice daily initial subconvulsive corneal stimulations (6Hz, 40mA, 3s) until the mice reach the 'fully kindled state', i.e. 10 consecutive generalized seizures. Seizures are scored each day using the scale of Racine (generalized seizure = score≥3). We observe that $xCT^{+/+}$ mice show significantly more severe seizures during this kindling process compared to $xCT^{-/-}$ mice. Subsequently we try to maintain the 'fully kindled state' by corneal stimulations twice a week. During this maintenance phase we also observed a trend of more severe seizures in the $xCT^{+/+}$ mice. These results are suggesting a somehow slight better outcome of $xCT^{-/-}$ mice, compared to their wildtype littermates, in the 6Hz corneal kindling model.

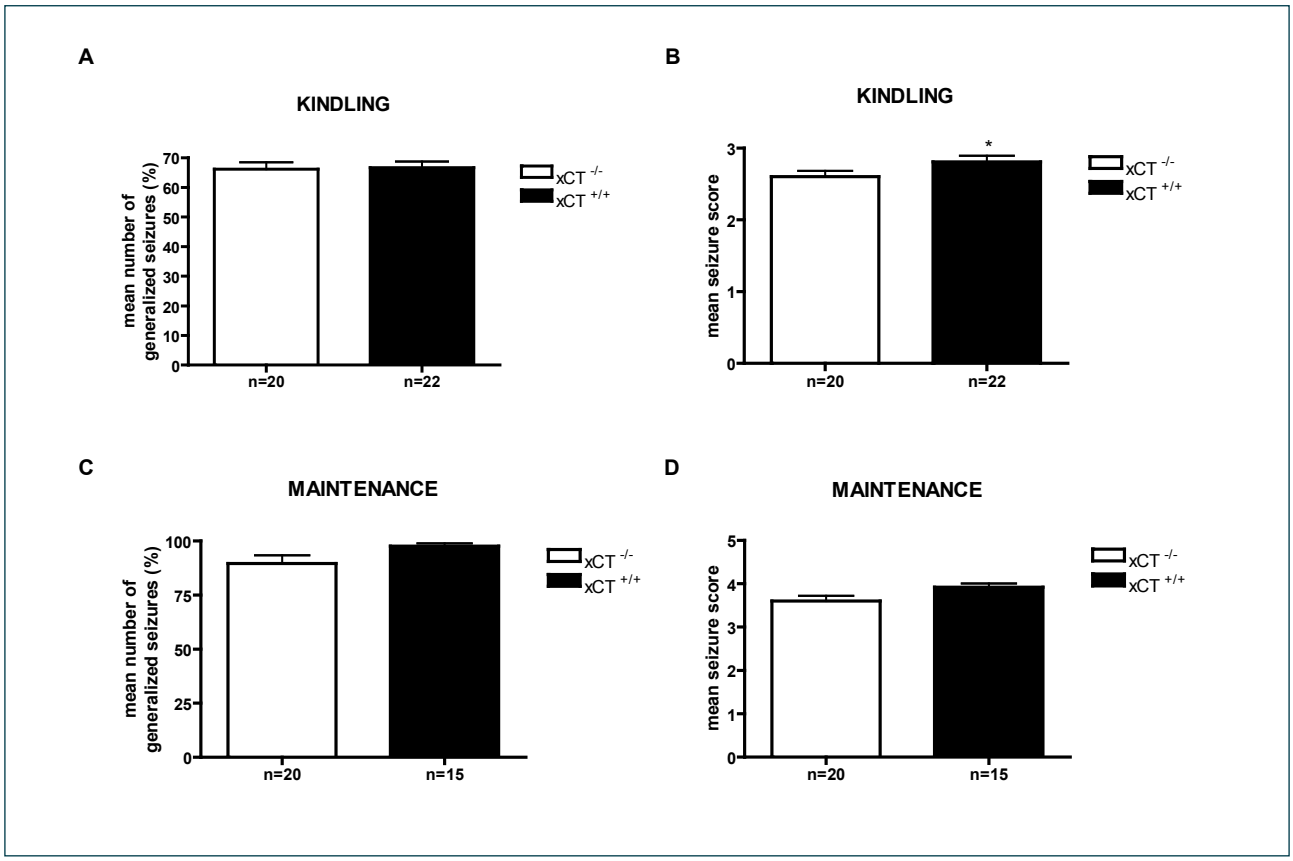


Figure 11. Preliminary data of 6Hz corneal kindling model using xCT$^{-/-}$ and xCT$^{+/+}$ mice. ***(A)*** *Mean number of generalized seizures during the kindling process (xCT$^{-/-}$ n=20 and xCT$^{+/+}$ n=22; Mann–Whitney U p=0.920).* ***(B)*** *Mean seizure score during the kindling process (xCT$^{-/-}$ n=20 and xCT$^{+/+}$ n=22; Mann–Whitney U p=0.034).* ***(C)*** *Mean number of generalized seizures during the maintenance phase (xCT$^{-/-}$ n=20 and xCT$^{+/+}$ n=15; Mann–Whitney U p=0.219).* ***(D)*** *Mean seizure score during the maintenance phase (xCT$^{-/-}$ n=20 and xCT$^{+/+}$ n=15; Mann–Whitney U p=0.096).*

4. 2011-2013 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.
  SCI impact factor = 7.115.
- J. Lewerenz, S.J. Hewett, Y. Huang, M. Lambros, P.W. Gout, P.W. Kalivas, **A. Massie, I. Smolders**, A. Methner, M. Pergande, S.B. Smith, V. Ganapathy & P. Maher.
  The Cystine/Glutamate Antiporter system x_c^- in Health and Disease: From Molecular Mechanisms to Novel Therapeutic Opportunities. Review.
  Antioxid Redox Signal. 18 (2013) 522-555.
  SCI impact factor 2012 = 7.189.
- J. Van Liefferinge, **A. Massie**, J. Portelli, G. Di Giovanni, **I. Smolders**.
  Are vesicular neurotransmitter transporters potential treatment targets for temporal lobe epilepsy?
  Front Cell Neurosci. 2013 Aug 30;7:139. Review.
  SCI Impactfactor 2012 = 4.469.
- J. Lewerenz, P. Baxter, R. Kassubek, P. Albrecht, J. Van Liefferinge, M.A. Westhoff, M.E. Halatsch, P.J. Meakin, G. Karpel-Massler, J.D. Hayes, E. Aronica, **I. Smolders**, A.C. Ludolph, A. Methner, M. Conrad, **A. Massie**, G.E. Hardingham & P. Maher
  Phosphoinositide 3-kinases upregulate system x_c^- via eIF2α and ATF4 – a pathway active in glioblastomas and epilepsy.
  Antioxid Redox Signal. 2013 Nov 12. [Epub ahead of print]
  SCI impact factor 2012 = 7.189.
- J. Van Liefferinge, E. Bentea, T. Demuyser, E. Mercks-x, K. Maes, H. Sato, **I. Smolders**, J. Lewerenz, **A. Massie**.
  Lack of effect of zonisamide treatment on glutathione levels and system x_c^-.
  Submitted to ***Annals of Neurology***

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.
  SCI impactfactor = 1.798.
- 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. 1436 (2012) 43-50.
  SCI Impactfactor = 2.879
- E. Loyens, D. De Bundel, H. Demaegdt, S.Y. Chai, P. Vanderheyden, **Y. Michotte**, P. Gard, **I. Smolders**.
  Antidepressant-like effects of oxytocin in mice are dependent on the presence of insulin-regulated aminopeptidase.
  Int J Neuropsychopharmacol. 16 (2013) 1153-1163.
  SCI Impactfactor 2012 = 5.641.

Final 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 aimed 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 aimed to pinpoint differential protein–protein interaction networks. In the project we also applied novel approaches to model CMT mutations, validated gene function and interaction networks. Importantly was our focus towards the study of small heat shock proteins involved in CMT and the hereditary distal motor neuropathy (HMN). In the GSKE reports of 2011 and 2012 we already highlighted our most relevant results, but added here our latest findings.

The special architecture of the peripheral nervous system, with axons extending for long distances, represents a major challenge for the intracellular transport system. We reported that mutations in the small heat shock protein HSPB1, which cause an axonal type of CMT neuropathy (HMN), affect microtubule (MT) dynamics and impede axonal transport (1). Intriguingly, while at presymptomatic age the neurons in the mutant HSPB1 mouse show a hyperstable MT network, at symptomatic age, the MT network completely lost its stability as reflected by a marked decrease in tubulin acetylation levels (2). We proposed a model explaining the role of MT stabilization and tubulin acetylation in the pathogenesis of *HSPB1* mutations (3). The remodeling capacity of MTs is essential for their proper function. In mammals, MTs are predominantly formed at the centrosome, but can also originate from non-centrosomal sites, a process that is still poorly understood. In 2013 we demonstrated that HSPB1 plays a role in the control of non-centrosomal MT formation. The HSPB1 expression level regulates the balance between centrosomal and non-centrosomal MTs. The HSPB1 protein can be detected specifically at sites of *de novo* forming non-centrosomal MTs, while it is absent from the centrosomes. In addition, we showed that HSPB1 binds preferentially to the lattice of newly formed MTs *in vitro*, suggesting that it functions by stabilizing MT seeds. Our findings opened new avenues for the understanding of the role of HSPB1 in the development, maintenance and protection of cells with specialized non-centrosomal MT arrays (4). Ten years ago we showed that mutations in HSPB1 disrupt neurofilament network and cause their aggregation (5). Furthermore, we and others have described mutations in the neurofilament light gene (*NEFL*) to be associated with axonal and demyelinating CMT phenotypes (6). We recently reviewed that neurofilaments (NFs) are the most abundant structural proteins in neurons and are thought to play a key role in neurodegeneration (7). Therefore, we investigated the effects of mutant HSPB1 on NF properties. We observed that transduction of neuronal cell lines with mutant HSPB1 affect the NFs axonal transport and binding to the anterograde motor protein kinesin. These deficits were also associated with an increased phosphorylation of NFs as well as an increased phosphorylation of Cdk5, which mediates the NF phosphorylation. To confirm the role of Cdk5 in this process, we showed in 2013 that inhibition of Cdk5/p35 restored the NF phosphorylation and binding to kinesin. Altogether, our findings suggest that specific mutations in HSPB1 induce hyperphosphorylation of NFs through Cdk5, resulting in a deficit in the axonal transport of NFs that could contribute to the axonal CMT phenotype (8).

In frame of this GSKE project we also contributed to the identification of novel genes and mutations for inherited peripheral neuropathies. For hereditary sensory and autonomic neuropathies (HSAN) we identified *SCN11A* (9), *ATL1* (10), *ATL3* (11) and *KIF1A* (12) as causal genes. We performed extensive

genotype-phenotype correlations in CMT families with known and novel mutations in *GDAP1* (13), *HINT1* (14), *BICD2* (15) and *INF2* (16). We reported the largest comprehensive study of the molecular architecture of peripheral neuropathies starting in the first year of life (17). Because of the rapid development of whole exome sequencing (WES), and the importance of pulling clinical data together from patients with these rare peripheral neuropathies, most of our these genetic studies were strongly embedded in international collaborations.

In summary, over the 3 year period our research resulted in 4 PhD's and 26 publications acknowledging the GSKE (see part 2).

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- Holmgren, A., Bouhy, D., **Timmerman, V.**: Molecular Biology of small HSPs associated with Peripheral Neuropathies. In: eLS. John Wiley & Sons, Ltd: Chichester. November (2012), DOI: 10.1002/9780470015902.a0024294, No IF yet
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- Bouhy,D., **Timmerman,V.**: Animal models and therapeutic prospects for Charcot-Marie-Tooth disease. Annals of Neurology 74(3): 391-396 (2013), IF = 11.19
- Holmgren,A., Bouhy,D., De Winter,V., Asselbergh,B., Timmermans,J-P., Irobi,J., **Timmerman,V.**: Charcot-Marie-Tooth causing HSPB1 mutations increase Cdk5-mediated phosphorylation of neurofilaments. Acta Neuropathologica 126(1): 93-108 (2013), IF = 9.73
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- **Timmerman,V.**, Clowes,V., Reid,E.: Overlapping molecular pathological themes link Charcot-Marie-Tooth neuropathies and hereditary spastic paraplegias. Experimental Neurology 246: 14-25 (2013), IF = 4.65
- Almeida-Souza,L., Asselbergh,B., De Winter,V., Goethals,S., **Timmerman,V.**, Janssens,S.: HSPB1 facilitates the formation of non-centrosomal microtubules. PLOS ONE 8(6): e66541 (2013), IF = 3.73
- Madrid,R.E., Löfgren,A., Baets,J., **Timmerman,V.**: Biopsy in a patient with PMP22 exon 2 mutation recapitulates Trembler-J pathology. Neuromuscular Disorders 23(4): 345-348 (2013), IF = 3.46
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Articles in Books:

- Landrieu,P., Baets,J., **De Jonghe,P.**: Chapter 146: Hereditary motor-sensory, motor, and sensory neuropathies in childhood. In: Handbook of Clinical Neurology - Pediatric Neurology Part III Edited by Dulac O., Lassonde M., Sarnat H.B. (Elsevier B.V.): 1413-1432 (2013)

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
- **P. De Jonghe and V. Timmerman:** Medical Foundation Queen Elisabeth, Prize Valine De Spoelberch, Brussels, Belgium, May 15, 2012
- **J. Baets:** Koninklijke Academie voor Geneeskunde van België, Prijs voor Klinisch Wetenschappelijk Onderzoek in de Geneeskunde 2012, 'Genotype-phenotype correlations in hereditary neuropathies: a systematic approach', November 17, 2012
- **J. Baets:** 'GlaxoSmithKline Prize 2012', Belgian Neurological Society, Brussels, January 18, 2013

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

- **J. Baets:** Peripheral Nerve Society, Arthur K. Asbury Travel Grant, 2012 PNS – INC Congress, Rotterdam, The Netherlands, June 24 – 27, 2012
- **J. Baets:** 2012 FWO travel grant, American Society of Human Genetics meeting, San Francisco, USA.
- **E. Ydens:** Peripheral Nerve Society, Rabobank Travel Grant, 2012 PNS-INC Congress, Rotterdam, The Netherlands, June 24-27, 2012
- **J. Baets:** FWO travel grant for longer research stay abroad, Queen Square MRC, July-September 2013, UK, London

PhD theses:

- **J. Baets: "**Genotype-phenotype correlations in hereditary neuropathies: a systematic approach", Promotors: 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", Promotors: Timmerman V. & Janssens S., December 13th 2011
- **A. Holmgren:** "Molecular biology of small heat shock protein mutations associated with Charcot-Marie-Tooth and distal hereditary", Promotors: Timmerman V. & Irobi J., September 10th 2012
- **M. Zimón:** Promotor: "Large scale genetic approach for the molecular characterization of autosomal recessive Charcot-Marie-Tooth Disease", Promotors: De Jonghe P. & Jordanova A., October 11th 2013

Master theses:

- **J. De Cleir:** "Effect van RAB7- en SPTLC2-mutaties op axonaal transport en axonale degeneratie", Supervisor: Janssens K. (Academic MSc Thesis Biochemistry & Biotechnology, UA)
- **T. Geuens:** "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)
- **L. Peeraer:** "De rol van TLR1 bij acute neurodegenerative in het perifere zenuwstelsel", Supervisors: Janssens S. & Timmerman V. (Academic MSc Thesis Biomedical Sciences, UA)
- **S. Vermeylen:** "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)
- **S. Vermeylen:** "Search for protein interactors of SH3TC2, a protein mutant in Charcot-Marie-Tooth neuropathy", Supervisors: F. Palau, V. Timmerman (Academic MSc Thesis Biochemistry & Biotechnology, Erasmus Valencia)
- **M. Krols:** "The role of mammalian Target Of Rapamycin (mTOR) in Schwann cell development and peripheral myelination", Supervisors: P. Brophy P, V. Timmerman (Academic MSc Thesis Biochemistry & Biotechnology, Erasmus Edinburgh)
- **E. Cottenie:** "Investigation of hereditary axonal neuropathies", Supervisors: M. Reilly, P. De Jonghe (Academic MSc Thesis Biochemistry & Biotechnology, Erasmus London)
- **D. Atkinson:** "Characterization of a *Drosophila* model for Charcot-Marie-Tooth type 2B", Supervisor: K. Janssens, Technician: B. Asselbergh (Academic MSc Thesis Biochemistry & Biotechnology, UA)
- **E. Adriaenssens: "**Molecular characterization of autophagy in Charcot-Marie-Tooth neuropathy caused by small heat shock protein mutations", Supervisors: D. Bouhy, V. Timmerman (Academic MSc Thesis Biochemistry & Biotechnology, UA)
- **A. Van Hauwermeiren: "**Treatment approaches of mouse models for charcot-marie-tooth disease via modulation of the immune system**",** Supervisors: R. Martini, V. Timmerman (Academic MSc Thesis Biochemistry & Biotechnology, Erasmus Würtzburg)

Chair and organizational activities:

- **V. Timmerman and P. De Jonghe:** Inherited Neuropathies Consortium (INC), Rare Disease Clinical Research Consortium (RDCRC), EAB meeting in Miami, USA, February 15-17, 2012, Member of the External Advisory Board
- **V. Timmerman and P. De Jonghe:** 5th International Charcot-Marie-Tooth Consortium meeting, organization and chair, Antwerp, Belgium, June 25-27, 2013

Invited Lectures at international meetings:

- **P. De Jonghe:** "CMT with early onset", 9th Congress of the European Paediatric Neurology Society; Cavtat, Croatia, May 11-14, 2011.
- **V. Timmerman:** "Molecular Genetics of Charcot-Marie-Tooth neuropathies", Italian Institute of Technology (IIT), seminar upon invitation by Dr. M. Pennuto, Genova, Italy, March 2, 2011

- **V. Timmerman:** "New molecular targets in hereditary neuropathies", UK Neuromuscular Translational Research Conference 2011, MRC Center for Neuromuscular Disease, London, UK, March 29-30, 2011
- **V. Timmerman:** 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
- **V. Timmerman:** "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
- **V. Timmerman:** "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
- **V. Timmerman:** "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
- **V. Timmerman:** "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
- **P. De Jonghe:** "Hereditary Neuropathies, an overview". 4. Neuromuskuläres Symposium, Neuropathien bei Kindern und Erwachsenen', Zürich, November 22, 2012 (Plenary Lecture)
- **V. Timmerman:** "Understanding the pathomechanisms of Charcot-Marie-Tooth neuropathies", Thomas Wahlig Foundation, Köln, Germany, March 16, 2012 (Plenary Lecture)
- **V. Timmerman:** "Understanding the pathomechanisms of inherited peripheral neuropathies", European Society of Human Genetics (ESHG) meeting 2012, Nürnberg, Germany, June 24-26, 2012 (Educational Lecture)
- **V. Timmerman:** "Modèles animaux dans la maladie de Charcot-Marie-Tooth et applications de la compréhension de la maladie chez l'homme", Journées de la Société de Neurologie, Paris, France, January 26, 2013
- **V. Timmerman:** "Small heat shock protein mutations causing Charcot-Marie-Tooth neuropathies", Cologne Spring Meeting, Cologne, Germany, February 28, 2013
- **V. Timmerman:** "Understanding disease mechanisms in Charcot-Marie-Tooth neuropathies", Deutsche Gesellschaft für Muskelkranke. Aachen, Germany, March 2, 2013
- **V. Timmerman:** "Inherited motor neuronopathies", Terza Riunione Annuale dell' Associazione Italiana per lo Studio del Sistema Nervoso Periferico, Verona, April 18-19, 2013
- **V. Timmerman:** "Understanding the pathomechanisms of inherited peripheral neuropathies", World Congress of Neurology 2013, Vienna, September 21-26, 2013
- **P. De Jonghe**: "Meet the Expert (Hereditary Neuropathies)", 10th European Paediatric Neurology Congress, Brussels, Belgium, September 25-28, 2013

Slide presentations selected at international meetings:

- **L. Almeida-Souza:** "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
- **J. Baets:** "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
- **J. Baets:** "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
- **A. Holmgren:** "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
- **F. Ipek:** "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
- **J. Irobi**: "Mutant HSPB8 causes motor neuron specific neurite degeneration", 4th international CMT consortium meeting, Potomac, MD, USA, June 29 – July 1, 2011
- **E. Ydens:** "Acute neurodegeneration triggers an alternative macrophage response", 91st Meeting of the Belgian-Dutch Neuromuscular Study Club, Sint-Lambrechts-Woluwe, Belgium, September 28, 2011
- **J. Baets:** "Autosomal Recessive Axonal Neuropathy with Neuro-Myotonia: a novel disease entity caused by mutations in HINT1", American Society of Human Genetics, San Francisco, USA, November 6-10, 2012
- **J. Baets:** "Autosomal recessive axonal neuropathy with neuromyotonia: a new disease entity", Peripheral Nerve Society satellite meeting, Rotterdam, The Netherlands, June 24-27, 2012
- **E. Ydens:** "Macrophage activation in Wallerian degeneration", Peripheral Nerve Society – Inflammatory Neuropathy Consortium (PNS-INC), Rotterdam, The Netherlands, June 24-27, 2012
- **D. Bouhy**: "Inherited peripheral neuropathies: an integrative approach", Neuromics Kick-off Meeting, Stiges, Spain, January 25-27, 2013

- **M. Krols:** "Quantifying ER-mitochondria contact points using 3D-EM imaging", EMBL BioImage Data Analysis course, Heidelberg, Germany, May 14, 2013
- **E. Ydens:** "The role of NOD-like receptors in peripheral nerve injury", PNS meeting 2013, Saint-Malo, France, June 29-July 3,2013

Poster presentations at international meetings:

- **A. Holmgren: "**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
- **K. Janssens:** "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
- **E. Ydens:** "Acute neurodegeneration triggers an alternative macrophage response, 10th European meeting on Glial Cells in Health and Disease", Czech Republic, Prague, September 13-17, 2011
- **B. Asselbergh:** "Peripheral neuropathy mutants stabilize microtubules and reveal a novel role for HSPB1 in microtubule nucleation", Novel Biophysical Approaches in the Investigation of the Cytoskeleton, the 27th European Cytoskeleton Forum Meeting, Pécs, Hungary, November 3-7, 2012**F.M. Ipek:** "Modelling a sensory neuropathy caused by mutations in SPTLC2 in Drosophila", GRC: Glycolipid and sphingolipid biology, Lucca, Italy, April 22-27, 2012
- **F.M. Ipek:** "Modelling a sensory neuropathy caused by mutations in SPTLC2 in Drosophila", Neurofly, Padova, Italy, September 3-7, 2012
- **K. Janssens:** "Modelling CMT2B, caused by mutations in RAB7A, in Drosophila melanogaster", Rab GTPases and their interactors in health and disease, Cork, Ireland, June 11-13, 2012
- **E. Ydens:** "Macrophage activation in Wallerian degeneration", Peripheral Nerve Society – Inflammatory Neuropathy Consortium (PNS-INC), Rotterdam, The Netherlands, June 24-27, 2012
- **V. Timmerman**: "Charcot-Marie-Tooth causing HSPB1 mutations increase Cdk5-mediated phosphorylation of neurofilaments", Peripheral Nerve Society Meeting, Saint-Malo, France, June 29 - July 3, 2013
- **V. Timmerman**: "HSPB1 facilitates the formation of non-centrosomal microtubules", Leonardo Almeida-Souza, Bob Asselbergh, Vicky De Winter, Sofie Goethals, Vincent Timmerman, Sophie Janssens. Peripheral Nerve Society Meeting, Saint-Malo, France, June 29 - July 3, 2013
- **D. Bouhy**: "Autophagy deficits: common pathomechanisms leading to peripheral Neuropathy?", EMBO Conference on Autophagy: molecular mechanism, physiology & pathology, Trollfjord, Norway, May 5-9, 2013.
- **D. Bouhy**: "Modelling Charcot-Marie-Tooth disease to unravel pathomechanisms", NeurOmics Joint Kick-off Meeting, Stiges, Spain, January 25-27, 2013.
- **M. Krols:** "Quantifying ER-mitochondria contact points using 3D-EM imaging", EMBL BioImage Data Analysis course, Heidelberg, Germany, May 13-17 2013
- **I. Mademan:** "Next Generation Sequencing and Hereditary Sensory and Autonomic Neuropathies: a proof of concept study", Genomic Disorders 2013, Cambridge, UK, April 11, 2013
- **K. Smets:** "AFG3L2 partial deletions can cause spinocerebellar ataxia type 28", SPATAX meeting, Paris, France June 12, 2013
- **E. Ydens:** "Characterization of macrophage populations in the peripheral nerve", CMT consortium 2013, Antwerp, Belgium, June 25-27, 2013
- **E. Ydens:** "The role of NOD-like receptors in peripheral nerve injury", CMT consortium 2013, Antwerp, Belgium, June 25-27, 2013
- **E. Ydens:** "The role of NOD-like receptors in peripheral nerve injury", PNS meeting 2013, Saint-Malo, France, June 29-July 3, 2013
- **E. Ydens:** "Characterization of macrophage populations in the peripheral nerve", PNS meeting 2013, Saint-Malo, France, June 29-July 3, 2013

Invited lectures at national meetings:

- **V. Timmerman**: "Recente resultaten uit het CMT onderzoek en mogelijkheden voor de toekomst", VSN congres, Veldhoven, Nederland, September 15, 2012
- **V. Timmerman:** "Understanding the pathomechanisms of inherited peripheral neuropathies", Research seminar at GIGA, Liège, January 10, 2013
- **J. Baets:** "What's new in neurology: genetic testing in neuropathies", 1st Joint BNS-VVN Meeting, Brussels, April 20, 2013

Slide presentations selected at national meetings:

- **A. Holmgren:** "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
- **J. Irobi:** "Mutant HSPB8 causes motor neuron specific neurite degeneration", 9th bi-annual Meeting of the Belgian Society for Neuroscience, Leuven, May 23, 2011
- **K. Janssens:** "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
- **L. Almeida-Souza:** "Hyperactivity and microtubule network stabilization underlies the pathogenesis of HSPB1 mutations in Charcot-Marie-Tooth neuropathy", VIB Seminar 2012, Blankenberge, Belgium, April 18-20, 2012
- **I. Mademan:** "Whole Exome Sequencing in CMT: waarom en is dit wel nuttig?" CMT jaarlijkse studie- en contactdag, Wilrijk, Belgium, October 27, 2012
- **L. Peeraer:** "Geïnduceerde pluripotente stamcellen voor CMT, waarom en is dit wel nuttig?" CMT studie- en contactdag, Antwerp, Belgium, October 27, 2012
- **E. Ydens:** "Acute neurodegeneration in the peripheral nervous system triggers an alternative macrophage response", VIB Seminar 2012, Blankenberge, Belgium, April 18-20, 2012
- **D. Bouhy:** "Neuroinflammation in the injured central and peripheral nervous systems", Symposium in honor of pr. J. Schoenen, Liège, September 14, 2013
- **D. Bouhy:** "Modeling inherited peripheral neuropathies: hints and caveats from animal and cell models", Autumn Meeting of The Belgian Society of Cellular Biology and Development Liège, October 19, 2013
- **K. Smets:** "Hereditary Spastic Paraparesis in Childhood and adulthood", LOK meeting, neurologists, Deurne - Monica Hospital, Antwerp, June 5, 2013
- **K. Smets:** "Hereditary Spastic Paraparesis in Childhood and adulthood", Journal Club, Neurologists, University Hospital of Antwerp, Neurology Department, Antwerp, July 19, 2013

Poster presentations at national meetings:

- **L. Almeida-Souza:** "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
- **F. Ipek:** "Modeling a sensory neuropathy caused by mutations in SPTLC2 in *Drosophila*", IAP P6/43 Meeting, Sint-Lambrechts-Woluwe, October 24, 2011
- **E. Ydens:** "Acute neurodegeneration triggers an alternative macrophage response", IAP P6/43 Meeting, Sint-Lambrechts-Woluwe, October 24, 2011
- **B. Asselbergh:** "An automated image analysis procedure to measure neurite outgrowth on phase contrast images of neurons in culture", 12th International meeting on advanced light microscopy, Leuven, Belgium, June 5-8, 2012
- **E. Ydens:** "NOD-like receptor expression in the peripheral nervous system", VIB Seminar 2013, Blankenberge, February 6-9, 2013
- **D. Bouhy:** "Mutant HSPB1 induces Cdk5 dependent hyperphosphorylation of neurofilaments: a new pathomechanism underlying CMT", VIB Seminar 2013, Blankenberge, February 6-9, 2013
- **D. Bouhy:** "Charcot-Marie-Tooth causing HSPB1 mutations increase Cdk5-mediated phosphorylation of neurofilaments", Fifth European and North American CMT consortium Meeting, Antwerp, June 25-27, 2013
- **M. Krols:** "Quantifying ER-mitochondria contact points using 3D-EM imaging", 5th international CMT consortium meeting, Edegem, June 25-27, 2013
- **I. Mademan:** "Next Generation Sequencing and Hereditary Sensory and Autonomic Neuropathies: a proof of concept study", VIB Seminar 2013, Blankenberge, February 6-9, 2013
- **I. Mademan:** "De novo INF2 mutations expand the genetic spectrum of Charcot-Marie-Tooth disease with focal segmental glomerulosclerosis", Fifth European and North American Charcot-Marie-Tooth Consortium Meeting 2013, Antwerpen, June 25-27

Societal activities at national meetings:

- **D. Bouhy:** "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
- **V. Timmerman:** "Staan onderzoek en ontwikkelen van medicatie los van elkaar?", Nationale Studie- en Contactdag van CMT België v.z.w, Antwerpen, March 2, 2011
- **P. De Jonghe**: "HSP, kliniek en behandeling", Patiëntenvereniging Strümpel-Lorrian, Beveren, Belgium, May 26, 2013

Final report of the research group of

Dr. F. Tissir

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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 ability to signal by homophilic and/or heterophilic interactions. 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, abdominal bristles and the facets of the eye. These structures are organized in the plane of epithelium, orthogonal to the apical-basal polarity axis, by a process referred to as planar cell polarity (PCP). During the wing hair development, PCP is characterized by accumulation of Celsr/flamingo at cell boundaries. Celsr/flamingo triggers polarity by selectively recruiting frizzled to one side and van gogh to the opposite side of the cell.

When we started studying the mammalian Celsr genes in early 2000s, two members (*Celsr1* and *Celsr2*) were listed in databases, but little (if any) was known about their functions. We identified the third member (*Celsr3*), explored the expression patterns of the three genes and inactivated them in mice. Our analyses show that they are widely expressed in the nervous system where they play crucial roles in neural tube closure, neuronal migration, ependymal polarity, and axon guidance (reviewed in (Boutin et al., 2012; Tissir and Goffinet, 2010; Tissir and Goffinet, 2013)).

During the 3 years of this project, we have focussed mainly on the role of Celsr genes in ependymal polarity and ciliogenesis, and axon guidance.

Celsr1-3 in ependymal polarity and ciliogenesis

The wall of the lateral ventricles of the postnatal forebrain, hereafter referred to as lateral wall “LW”, is a region where neural stem cells coexist with ependymal cells. A striking feature of multiciliated ependymal cells is their high degree of polarization. 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). This specific organization of the lateral wall is essential for cerebrobrospinal fluid (CSF) circulation and its modification is thought to affect stem cell maintenance and adult neurogenesis.

In 2010, we showed that Celsr2 and Celsr3 impairs ciliogenesis and leads to defective CSF flow and lethal hydrocephalus. Mutant ependymal cilia never develop in normal numbers and display abnormalities in morphology, position, and planar organization. Ciliary basal feet are mis-oriented, and basal bodies were seen ectopically deep in the cytoplasm. The conventional method to analyze rotational polarity is to investigate the orientation of the basal foot by transmission electron microscopy. This method is time consuming. To speed up studies of LW in our mutants, we developed an alternative approach wherein we combined immunostaining and confocal microscopy. Gamma tubulin and phospho-beta-catenin localize at opposite sides of the BB and define a vector which nicely delineates rotational polarity of cilia. Using this method, we expanded on our initial finding and show that Celsr2 and Celsr3, together with

Fzd3 and Vangl2, control not only the orientation of motile cilia but also their spacing and heir lattice organization in individual cells.

To investigate the potential role of Celsrs 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 WT and PCP mutants, ependymal cells showed a displacement of cilia. However, while in WT animals, all ciliary tufts are systematically shifted toward the anterior pole, *Celsr1* mutant mice display abnormal translational polarity with cilia dispersed in any pole of ependymal cells. It has been suggested that the primary cilium of radial glial (RG) cells control the translational polarity of multicilia. We analyzed the presence of primary cilium in our mutants. Immunostaining against either gamma tubulin or acetylated tubulin demonstrated that virtually all RG cells bear a primary cilium at birth suggesting that the translational polarity defects observed in *Celsr1* 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, the primary (mono) cilium 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*, and Vangl2. Interestingly RG polarity is not affected in *Celsr2* or *Celsr3* mutants. Our results show that *Celsr1*, *Fzd3* and *Vangl2* position the primary cilium in radial progenitors. In ependymal cells, whereas *Celsr2&3*, *Fzd3* and *Vangl2* work together to organize cilia tufts in a given cell; *Celsr1*, *Fzd3* and *Vangl2* coordinate polarity between cells. These signals are relayed by distinct cytoskeletal changes. These data reveal unreported functions of Celsr genes and PCP signaling and provide an integrated view as how polarity is set in radial progenitors and passed on to ependymal. In addition to the published articles listed below, the rest of the data has been compiled in a manuscript that is under revision in *PloS Biology*. The pdf file is attached (appendix 1)

Celsr3 in wiring of the nervous system

Since our initial report (Tissir et al., 2005), we and others have accumulated evidence that Celsr3 is a major player in directional growth of axons and wiring of the central nervous system(Feng et al., 2012; Fenstermaker et al., 2010; Lewis et al., 2011; Onishi et al., 2013; Price et al., 2006; Zhou et al., 2008a; Zhou et al., 2010; Zhou et al., 2008b; Zhou et al., 2009a; Zhou et al., 2007; Zhou et al., 2009b). However, whether Clesr3 acts in collaboration with, or in parallel to, other axon guidance systems such Eph/ephrins, Slit/Robo, Semaphorins/Plexins was not known; and whether it is involved in wiring of the peripheral and the enteric nervous systems was not investigated.

The enteric nervous system (ENS) constitutes a network of interconnected ganglia, which are arranged radially throughout the gut and integrate local and systemic signals to control gastrointestinal motility, secretion, and blood flow. In vertebrates, the majority of enteric neurons and glia are derived from neural crest cells which invade the foregut and, migrating rostro-caudally, colonize the entire length of the gastrointestinal tract. Considerable progress has identified a number of signaling pathways that control the migration of ENS progenitor cells and their differentiation into enteric neurons. However, the organizing principles of enteric connectivity and the mechanisms underlying the assembly of functional circuits from differentiated enteric neurons remain poorly understood.

In a collaborative study with the groups of Pieter Vanden Berghe (KUL, Leuven) and Vassilis Pachnis (MRC, London UK), we have combined in vivo and ex vivo physiological assays with gene inactivation and single-cell labeling to demonstrate that, in mice, Celsr3, together with Fzd3, controls gastrointestinal function by regulating the spatial organization of neuronal processes during gut organogenesis and the connectivity of ENS. In control guts, the vast majority of identifiable neuronal processes were directed anally parallel to the longitudinal axis. However, in both *Celsr3* and *Fzd3* mutants, a significantly larger fraction of neural projections were arranged circumferentially or directed orally. In addition to the altered trajectory, Celsr3- and Fzd3-deficient enteric neurons had shorter primary neurites, while a fraction of them acquired bipolar or multipolar morphology. Conditional inactivation of Celsr3 upon crossing Celsr3 floxed allele with Wnt-Cre transgenic mice demonstrated that Celsr3 is required cell autonomously in neural crest derivatives for normal development of ENC and for gastrointestinal function. Taken together, our studies identify Celsr3 as a critical regulator of ENS wiring in vivo and provide insight into the connection pathology that might underlie some gut motility disorders. This work was published in Journal of Clinical Investigation en 2013.

To probe the role of Celsr3 in the peripheral nervous system, we studied the consequences of its loss-of-function on limb innervation. We found that mice with conditional inactivation of Celsr3 in motor neurons often exhibit uni- or bilateral paralysis of the hindlimb. Muscles of the anterior compartment of the hindlimb, particularly the tibialis anterior, are very atrophic, pointing to a defect of peripheral motor innervation that was confirmed by electrophysiology. Further studies showed that *Celsr3* mutants have a selective deficit of innervation of extensor muscles innervated by the dorsal, peroneal nerve, whereas axons of tibial nerve that innervate ventral muscles are unaffected. Fzd3 mutants have an identical phenotype. EphA4 mutant mice as well as mice with inactivation of the GDNF receptor components Ret and GFRa1 have a similar phenotype, namely absence of dorsal peroneal nerve, with rerouting of axons ventrally, hinting at possible interactions between Celsr3/Fzd3 and those two important signals.

Detailed phenotype analysis showed that, in *Celsr3* mutant mice, axons of the peroneal nerve segregate from those of the tibial nerve, but fail to extend dorsally and stall near the branching point. Those axons are not rerouted ventrally; thus, the phenotype is not identical to that in *EphA4* and *GDNF* mutant animals. *Celsr3* mutant axons respond to the repulsive signal generated by ephrinsAs expressed in the ventral limb mesenchyme acting on EphA4 in motor neurons. They are also able to respond to the attractive signal of GDNF. By contrast, Celsr3 and Fzd3 mutant motor neurons, contrary to as the wildtype axons, are not attracted by EphA-Fc in the Dunn chamber assay. This clearly shows that Celsr3 and Fzd3-deficient axons are no longer able to respond to the reverse ephrin signaling triggered by EphA expressed in dorsal limb mesenchyme acting on ephrin A receptors in growth cones. Using *EphA4* mutant mice, we showed that Celsr3 interacts genetically with EphA4. We also demonstrated that Celsr3 associates physically with ephrinA2 and A5 in transfected HEK cells, and that Celsr3 co-immunoprecipitates with Fzd3 as predicted, as well as with Ret and GFRa1. Intriguingly, the peripheral axonal phenotype was not seen in mice with inactivation of the core planar polarity gene Vangl2, and no physical interaction between vangl2 and Celsr3 was detected, indicating that Celsr3 and Fzd3 regulate axon guidance in a Vangl2 independent manner. Our results provide strong evidence that Celsr3/Fzd3 interact with EphA:ephrinA reverse signaling to guide motor axons in the hindlimb.
This work is under revision in *Nature Neuroscience*. The pdf file of the manuscript is attached (appendix 2)

Peer-reviewed publications 2011-2013

2013:

- Onishi K, Shafer B, Lo C, **Tissir F**, **Goffinet AM**, Zou Y.
  Antagonistic functions of Dishevelleds regulate Frizzled3 endocytosis via filopodia tips in Wnt-mediated growth cone guidance.
  Journal of Neuroscience 33:19071-19085.
  IF : 6.91
- Tatin F, Taddei A, Weston A, Fuchs E, Devenport D, **Tissir F**, MakinenT.
  Planar cell polarity protein Celsr1 regulates endothelial adherens junctions and directed cell rearrangements during lymphatic valve morphogenesis.
  Developmental Cell 26: 31-44 (2013)
  IF: 14.03
- **Tissir F** & **Goffinet AM**.
  Shaping the nervous system: Role of the planar cell polarity genes.
  Nature Reviews Neuroscience 14: 525-535 (2013)
  IF: 30.45
- **Tissir F** & **Goffinet AM**.
  Atypical cadherins Celsr1-3 and planar cell polarity in vertebrates. The Molecular Biology of Cadherins, Edited by Franz van Roy; *Progress in Molecular Biology and Translational Science* 116: 193-214 (2013)
  (book chapter)
- Sasselli V, Boesmans W, Vanden Berghe P, **Tissir F**, **Goffinet AM**, Pachnis V.
  Planar cell polarity genes control the connectivity of enteric neurons.
  Journal of Clinical Investigation 123: 1763-1772 (2013)
  IF: 15.43
  Vardarajan B*****, Vergote D*****, **Tissir F***, Logue M, Yang J, Daude N, Ando K, Rogaeva E, Lee J, Cheng R, Brion JP, Ghani M, Shi B, Baldwin CT, Kar S, Mayeux R, Fraser P, Goffinet AM, George-Hyslop PS, Farrer LA, Westaway D.
  Role of p73 in Alzheimer disease: lack of association in mouse models or in human cohorts.
  Molecular Degeneration 8: 1-14 (2013)
  ***** : Equal contribution.
  IF: 4.01

2012:

- Boutin C, Goffinet AM, **Tissir F**.
  Celsr1-3 Cadherins in PCP and Brain Development.
  Current Topics in Developmental Biology 2012 ;101:161-183.
  IF: 6
- Feng J, Xu Y, Wang M, Ruan Y, So KF, **Tissir F**, Goffinet A, Zhou L.
  A role for atypical cadherin Celsr3 in hippocampal maturation and connectivity.
  Journal of Neuroscience 2012; 32(40):13729-13743
  IF: 7.11
- **Tissir F**, **Goffinet AM**.
  Cilia: conductors' batons of neuronal maturation.
  Nature Neuroscience 2012; 15(3):344-345.
  IF: 15.25
- Pietri S, Dimidschstein J, Tiberi L, Sotiropoulou PA, Bilheu A, **Goffinet A**, Achouri Y, **Tissir F**, Blanpain C, Jacquemin P, Vanderhaeghen P.
  Transcriptional Mechanisms of EphA7 Gene Expression in the Developing Cerebral Cortex. *Cerebral Cortex* 2012; 22(7):1678-89.
  IF: 6.28
- Cortijo C, Gouzi M, **Tissir F**, Grapin-Botton A. Planar Cell Polarity Controls Pancreatic Beta Cell Differentiation and Glucose Homeostasis.
  Cell Reports 2012; 2:1593-1606.
  IF: Unknown. *Cell Reports* is a new journal launched in 2012 by Cell press. The "predicted" IF is around 8 according to the number of articles and citations in 2012.

2011:

- **Tissir F**, **Goffinet AM**. p73 and p63: Estranged relatives?
  Cell Cycle 10:1351 (2011)
  IF: 5.35

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

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

Inflammatory responses are regulated by intracellular signalling pathways initiated by the activation of innate immune receptors and cytokine receptors. A crucial transcription factor controlling inflammatory responses is NF-κB. **Although NF-κB activation in peripheral immune cells is absolutely essential for the induction of EAE pathology, little is still known about the involvement of NF-κB in the inflammatory reactions locally in the CNS** (Mc Guire *et al*., 2013a)**.** However, we could previously 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), as well as in a second mouse model for CNS demyelination induced by the neurotoxicant cuprizone (Raasch, van Loo *et al*., 2011).

1. A20/TNFAIP3 in central nervous system inflammation

As NF-κB activation is so crucial in many biological cellular processes, it is not surprising that a tight regulation of the pathway and the genes induced is an absolute requirement. For this, cells employ a multilayered control system to keep immunity and inflammation in check, and the combined action of different positive and negative regulators help to fine-tune the immune response. **One critical brake on NF-κB activation is A20/TNFAIP3** (TNFα induced protein 3). A20 is a cytoplasmic zinc finger protein that has been characterized as a dual inhibitor of NF-κB activation and apoptosis (Catrysse *et al.*, 2013). In most cell types, A20 expression is very low without stimulation but is rapidly transcriptionally induced by NF-κB. Once expressed, A20 functions as a negative feedback regulator of NF-κB activation. The essential role of A20 in the regulation of NF-κB and apoptotic signalling was demonstrated through the generation of a complete A20 knockout mouse (Lee *et al*., 2000). Mice deficient for A20 develop severe inflammation and cachexia, are hypersensitive to LPS and TNF, and die prematurely. A20-deficient cells fail to terminate TNF-induced NF-κB responses and are more susceptible to TNF-mediated apoptosis. Besides its critical role for the regulation of TNF-receptor-dependent pro-inflammatory signals, A20 is also required for termination of Toll-like receptor (TLR) and Nucleotide-binding Oligomerization Domain containing 2 (NOD2) receptor responses. Interestingly, *A20/TNFAIP3* has been identified in humans as a susceptibility locus for multiple immunopathologies including Crohn's disease, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (reviewed by Vereecke *et al.,* 2009; Catrysse et al.,

2013). Importantly, we could recently confirm these associations using mice with a conditional A20 knockout allele, allowing tissue-specific A20 deletion (Vereecke *et al*., 2010; Kool *et al.,* 2011; Matmati *et al*., 2011). These findings clearly indicate a crucial and cell type specific role for A20 in controlling inflammatory immune responses. **Interestingly, genome-wide association studies also suggested *A20/TNFAIP3* as a susceptibility gene for multiple sclerosis** (De Jager *et al*., 2009; Gilli *et al.,* 2011).

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

1.1 A20/TNFAIP3 in MS/EAE pathology

To study the CNS-specific role of A20 in the immunopathology of MS, we make use of the experimental MS model EAE, which can be induced by immunization of mice with myelin oligodendrocyte glycoprotein (MOG) or other encephalitogenic agents. To evaluate the cell specific contribution of A20 in the pathogenesis of EAE, different cell type-specific A20 knockout mice (all-CNS-, neuron-, astrocyte-, oligodendrocyte-, and microglia-specific, as well as T cell-, myeloid cell- and DC-specific) were generated from a conditional 'floxed' A20 knockout line (Vereecke *et al*., 2010) crossed to transgenic Cre lines which express the Cre cDNA under control of a cell type-specific promotor (respectively NestinCre, Thy1.2Cre, GFAPCre, MOGiCre, Cx3Cr1Cre, CD4Cre, LysMCre and CD11cCre). These cell type-specific A20 knockout mice and control littermates are then 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. Primary neuronal cultures are used *in vitro* to establish the impact of A20 deficiency on inflammatory challenge. Next to the EAE model of MS, brain-specific demyelination is also induced by putting mice on a diet containing the neurotoxicant cuprizone. In this model, administering cuprizone for 6 weeks causes complete demyelination of the corpus callosum in the absence of an immune reaction. Furthermore, when this administration is terminated, complete remyelination of the corpus callosum occurs, rendering this model useful to study both de- and remyelination. CNS demyelination, and astro- and microgliosis are evaluated by histology on corpus callosum sections, and inflammatory cytokine and chemokine expression is assessed by qPCR.

Although immune cell-specific contributions of A20 in EAE pathology could be assessed (Mc Guire *et al*., ongoing studies), no differences in disease development could be detected between CNS-specific A20 knockout mice and control wild-type littermates (Mc Guire *et al*., ongoing studies), in contrast to a recent study in which an astrocyte-specific contribution of A20 in EAE has been described (Wang *et al*., 2013).

1.2 A20/TNFAIP3 in cerebral ischemia

Cerebral ischemia is characterized by the activation of glial cells, causing a rapid and massive local inflammatory reaction leading to tissue damage and neuronal cell death (Ridder and Schwaninger, 2009). Over the past decade, it has become increasingly clear that NF-κB plays a central role in the pathogenesis of cerebral ischemia. NF-κB gets activated in the ischemic hemisphere shortly after permanent middle cerebral artery occlusion (MCAO). Furthermore, NF-κB deficient mice show a reduction in ischemic damage after transient or permanent MCAO, suggesting a cell death promoting role for NF-κB in this

model. In line with these, mice lacking IKK2 in all neuroectodermal cells or specifically in neurons show a decreased infarct volume 48 hours after pMCAO, whereas constitutive activation of IKK2 increased infarct size (Herrmann *et al.,* 2005). However, NF-κB may also act beneficial in conditions of ischemic preconditioning which was shown to protect against a subsequent prolonged ischemic insult through transcriptional activition of NF-κB.

Since A20 is a key negative regulator of NF-κB signaling, but can also act as a strong anti-apoptotic protein, we sought to clarify the *in vivo* role of A20 in the MCAO model of brain ischemia in mice. We demonstrated that NF-κB driven genes such as *A20, TNF* and *IL-6* are upregulated in the infracted area 24 hours post pMCAO. In agreement with these *in vivo* data, A20 mRNA was also upregulated in primary murine cortical neurons when placed in a glucose deprived 0,1% O_2 hypoxic environment for four hours *in vitro*. Since A20 seems to be differentially regulated both *in vivo* and *in vitro* during ischemic conditions, we questioned whether mice lacking A20 specifically in the CNS ($A20^{CNS\text{-}KO}$) or exclusively in neurons ($A20^{NEUR\text{-}KO}$) would be affected differently after pMCAO when compared to wild type littermates. $A20^{CNS\text{-}KO}$ or $A20^{NEUR\text{-}KO}$ mice, together with control littermate mice, were subjected to pMCAO for 24 hours after which the infarct size was estimated by means of a silver staining technique. To our surprise the infarct volume after 24 hours of pMCAO did not differ between $A20^{CNS\text{-}KO}$ or $A20^{NEUR\text{-}KO}$ and their respective wild type control littermates. Collectively, these results clearly demonstrate that, although A20 is upregulated in conditions of pMCAO, the lack of A20 in either all cells of neuroectodermal origin or neurons in particular does not influence the outcome of pMCAO in mice (Mc Guire *et al*., 2013b).

2. Mucosa-associated lymphoid tissue 1 (MALT1) in CNS inflammation

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

2.1 MALT1 in MS/EAE

Because of its essential role in T and B cell activation, MALT1 is considered an important therapeutic target in autoimmunity. However, so far its role in the development of autoimmune disease has not been reported. We sought to address the role of MALT1 in the generation of autoreactive T cells in the context of EAE. For this, we induced EAE in mice deficient in MALT1 ($MALT1^{-/-}$) and in wild-type and heterozygous littermate control mice ($MALT1^{+/+}$ and $MALT1^{-/+}$). $MALT1^{-/-}$ mice were completely protected from EAE, which was reflected in the absence of immune cell infiltration, demyelination and axonal damage in the spinal cord. Furthermore, splenocytes from $MALT1^{-/-}$ mice failed to produce an autoreactive T cell response and failed to induce autoimmune inflammation upon transfer in wild-type

mice. Finally, cleavage of the MALT1 substrates A20 and CYLD was shown in wild-type T cells from EAE diseased mice. Collectively, these data demonstrate a crucial role for MALT1 in T cell activation and in the early priming phase of EAE, suggesting that targeting MALT1 might be an important therapeutic strategy to treat MS (Mc Guire *et al*., 2013c).

2.2 Pharmacological inhibition of MALT1 protease activity in EAE

Very recently, small molecule inhibitors of MALT1 proteolytic activity were identified and shown to have promising anti-cancer properties in subtypes of B cell lymphoma (Fontan *et al.,* 2012; Nagel *et al*., 2012). However, information on the therapeutic potential of small compound inhibitors that target MALT1 protease activity in autoimmunity is still lacking. We studied the therapeutic potential of such a recently identified inhibitor of MALT1 protease activity, the phenothiazine derivative mepazine, in the context of EAE. We could demonstrate that administration of mepazine prophylactically or after disease onset, can attenuate EAE. Importantly, while complete absence of MALT1 affects the differentiation of regulatory T (Treg) cells *in vivo* (Mc Guire *et al*., 2013c), the MALT1 protease inhibitor mepazine did not affect Treg development. Together, these data indicate that small molecule inhibitors of MALT1 not only hold great promise for the treatment of B cell lymphomas but also autoimmune disorders such as MS (Mc Guire *et al*., in revision).

Conclusion

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

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From stem cells to cortical networks

State of the Art / Objectives.

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

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

We focused on the following main objectives:
1. Multiple roles for ephrin/Eph guidance genes in the development of the cortex.
2. Understanding the mechanisms of specification of cortical neurons.
3. Linking development and evolution of the human cortex.
4. Exploring new ways to repair the diseased cortex.

Results.

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

We previously demonstrated that ephrin/Eph genes are involved in several aspects of the development of the connectivity of the forebrain, including the patterning of cortical sensory areas and development of area-specific thalamo-cortical projections (Vanderhaeghen and Polleux, 2004; Dufour et al., 2003; Seibt et al., 2003; Egea et al., 2005; Dufour et al., 2006).
We have now pursued these findings by looking at the potential involvement of ephrin/Eph genes in the neuronal migration in the cortex. This has led to the demonstration that ephrins are required for the proper patterning of cortical columns (Dimidschstein et al., 2013).
Specifically we found that ephrin-B1 plays a key role in this process through the control of non-radial steps of migration of pyramidal neurons. *In vivo* gain of function of ephrin-B1 resulted in a reduction of tangential motility of pyramidal neurons, leading to abnormal neuronal clustering. Conversely, following genetic disruption of ephrin-B1, cortical neurons displayed a wider lateral dispersion, resulting in enlarged ontogenic columns. Dynamic analyses revealed that ephrin-B1 controls the lateral spread of pyramidal neurons by limiting neurite extension and tangential migration during the multipolar phase. Furthermore we identified P-Rex1, a guanine-exchange factor for Rac3, as a downstream ephrin-B1

effector required to control migration during the multipolar phase.
Our results demonstrate that ephrin-B1 inhibits non-radial migration of pyramidal neurons, thereby controlling the pattern of cortical columns.

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

- Dimidschstein, J., Passante, L., Dufour, A., van den Ameele, J., Tiberi, L., Hrechdakian, T., Adams, R., Klein, R., Lie, D.C., Jossin, Y., and **Vanderhaeghen P.** (2013). Ephrin-b1 controls the columnar distribution of cortical pyramidal neurons by restricting their tangential migration.
  Neuron *79*, 1123-1135.

2. Understanding the mechanisms of specification of cortical neurons.

Using our model of corticogenesis from mouse ES cells, we have performed a gain of function screen aimed at identifying novel transcriptional programmes involved in cortical neurogenesis.
About 20 transcription factors known to be expressed in the developing cortex but without a well characterized function, were overexpressed transiently during in vitro cortical neurogenesis, using in lab-generated ESC lines allowing inducible gene expression upon doxycyclin (Dox) addition (Pietri et al., 2012; van den Ameele et al., 2012). Among the genes tested, the BCL6 B cell oncogene (Ye et al., 1997; Ye et al., 1993) stood up markedly as a potent proneurogenic factor, that triggers the differentiation of cortical progenitors into pyramidal neurons (Tiberi et al., 2012a).
To validate these findings physiologically, we tested for the requirement of BCL6 during normal in vivo cortical neurogenesis, using BCL6 knock-out mice. This revealed striking defects in cell cycle exit of neural progenitors and impaired production of pyramidal neurons following BCL6 gene disruption. These complementary approaches thus lead to the important conclusion that BCL6 is necessary and sufficient to promote the generation of cortical pyramidal neurons. We then seeked for the molecular mechanism by which Bcl6 controls cortical neurogenesis, and found that BCL6 acts by direct repression of the transcription of Hes5, an essential target of the Notch pathway that maintains cortical progenitors undifferentiated (Kageyama et al., 2009). In addition, we found that the repression of Hes5 by BCL6 involves the recruitment of Sirt1 NAD+-dependent deacetylase (Herranz and Serrano, 2010), which triggers Histone deacetylation at the level of the hes5 promoter, and thereby stable epigenetic silencing during neurogenesis.
These data identify BCL6 as a novel and key actor in cortical neurogenesis, and uncover for the first time Notch/BCL6/Sirt1 interactions that may impact many other aspects of physiology and disease (Tiberi et al., 2012a).

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

- Tiberi L, van den Ameele J, Dimidschstein J, Piccirilli J, Gall D, Herpoel A, Bilheu A, Bonnefont J, Iacovino M, Kyba M, Bouschet T, and **Vanderhaeghen P**. 2012. BCL6 controls neurogenesis through Sirt1-dependent epigenetic repression of selective Notch targets.
  Nature Neuroscience 15:1627-1635.
- Pietri S, Dimidschstein J, Tiberi L, Sotiropoulou PA, Bilheu A, Goffinet A, Achouri Y, Tissir F, Blanpain C, Jacquemin P, and **Vanderhaeghen P**. 2012. Transcriptional Mechanisms of EphA7 Gene Expression in the Developing Cerebral Cortex.
  Cereb Cortex. 22:1678-1689.
- van den Ameele J, Tiberi L, Bondue A, Paulissen C, Herpoel A, Iacovino M, Kyba M, Blanpain C, and **Vanderhaeghen P**. 2012. Eomesodermin induces
  Mesp1 expression and cardiac differentiation from embryonic stem cells in the absence of Activin.
  EMBO Reports 13:355-362.

3. Linking development and evolution of the human cortex.

We have started to study the cellular and molecular properties of a novel model of corticogenesis from human ES cells recently developed in the lab, largely based on our previously described mouse ES model. Like in the mouse, human cortical-like progenitors are generated in a chemically defined medium, and cortical-like neurons (corresponding mostly to pyramidal neurons, the main subtype of cortical neurons) of diverse layers are generated in a time-dependent fashion. These pyramidal-like neurons can mature in vitro and make functional synapses with each other, as revealed by patch clamp experiments (Espuny-Camacho et al., 2013a).

In addition, we performed xenografting of these human cortical cells into the neonatal cortex of NOD/SCID mice, to test for their capacity to integrate in cortical networks in vivo. This revealed that the transplanted neurons can integrate for up to 12 months in the mouse, and develop elaborate and specific patterns of axonal and dendritic projections. Furthermore electrophysiology experiments revealed that the transplanted neurons display functional synapses with each other, and with the host brain. These important data demonstrate for the first time in vivo the cortical identity of human ES/iPSC-derived neurons based on their morphology and pattern of connectivity with their subcortical targets.

Our results thus show that corticogenesis can be efficiently achieved from hES and iPS cells, following a pathway that is similar to its murine couterpart, but that also presents interesting differences, some of which may have direct relevance to brain evolution (Espuny-Camacho et al., 2013b; Nagashima et al., 2013). Specifically, while mouse ES-corticogenesis takes about three weeks to be completed, it takes more than 10 weeks starting from human ES cells. In addition, the onset of neurogenesis appears much earlier in the mouse than in the human system, and is correlated with a different timing of appearance of neurogenic radial glia-like progenitors. Finally the timing of neuronal maturation is considerably slower with the human cells, as it takes up to six months for transplanted human neurons to develop mature patterns of axonal and dendritic growth, and synaptogenesis. Such differences are strikingly reminiscent of the properties of human corticogenesis (Fish et al., 2008; Kriegstein et al., 2006).

We have thus generated a unique experimental model to study cortical development with human cells (Espuny-Camacho et al., 2013a), which can now be used to study developmental mechanisms related to human brain evolution (such as the srGAP2 gene uncovered in (Charrier et al., 2012) or other evolutionarily related genes expressed that we identified in human cortex (Igoillo-Esteve et al., 2013; Lambert et al., 2011), and to model human neurodevelopmental disorders.

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

- Espuny-Camacho I, Michelsen K, Gall D, Linaro D, Hasche A, Bonnefont J, Bali C, Orduz D, Bilheu A, Herpoel A, Lambert N, Gaspard N, Péron S, Schiffmann SN, Giugliano M, Gaillard A, **Vanderhaeghen P**. 2013. Pyramidal neurons generated from human pluripotent stem cells integrate efficiently into mouse brain circuits in vivo. Neuron. 77:100-117.
- Nagashima, F., Suzuki, I.K., Shitamukai, A., Sakaguchi, H., Iwashita, M., Kobayashi, T., Tone, S., Toida, K., **Vanderhaeghen, P.**, and Kosodo, Y. (2013). Novel and robust transplantation reveals the acquisition of polarized processes by cortical cells derived from mouse and human pluripotent stem cells. Stem Cells Dev in press.
- Charrier C, Joshi K, Coutinho-Budd J, Kim JE, Lambert N, de Marchena J, Jin WL, **Vanderhaeghen P**, Ghosh A, Sassa T, Polleux F. 2012. Inhibition of SRGAP2 Function by Its Human-Specific Paralogs Induces Neoteny during Spine Maturation. Cell. 149:923-935.

4. Exploring new ways to repair the diseased cortex.

We have started to explore the relevance of ESC-derived in vitro corticogenesis for brain repair, using intracerebral grafting in experimental models of cortical lesions in the mouse.

To achieve this, we first implemented a well established experimental setup (Gaillard et al., 2007): focal neuronal lesions of the cerebral cortex were generated following sterotactic injections of ibotenic acid

neurotoxin, resulting in a focal loss of neurons in defined cortical domains, in frontal or occipital cortex. Three days after lesioning, Tau GFP ES-derived cortical progenitors and neurons (generated following (Gaspard et al., 2009) were grafted at the same site of the lesion.
Analysis of grafted animals 1-3 month after grafting indicated that most of them (80%) contained a graft, consisting mainly of differentiated pyramidal neurons. Most importantly, inspection of the rest of the brain revealed in 40% of the cases far-reaching graft-derived axonal growth, following specific paths and reaching specific targets of endogenous cortical neurons. Remarkably, we also found that the patterns of axonal growth were area-specific, i.e. ES-derived neurons with visual cortex identity and grafted in visual cortex send axons to visual and limbic targets, like in neonatal brain (Gaspard et al., 2008), but not following grafting in frontal cortex. These data indicate that ES-derived cortical neurons can display area-specific patterns of projections even in the adult brain, and that optimal restoration of cortical projections requires a precise match between the areal identity of the lesioned neurons and of the grafted neurons.

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

This part of the project is part of the following submitted publication:

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

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