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

Verslag – Rapport – Bericht – Report

2014

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

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Geneeskundige Stichting Koningin Elisabeth
2014
Inleiding verslag activiteiten van de GSKE – FMRE

Het jaar 2014 was het startjaar van een nieuwe driejaarlijkse cyclus (2014-15-16) voor de geselecteerde onderzoeksteams.

De Raad van Bestuur bevestigde het klassement dat werd voorgesteld door het Wetenschappelijk Comité en verleende de som van 40.000 euro per jaar gedurende 3 jaar, voor zover de financiële situatie het toelaat, aan de volgende teams: prof. dr. C. Bagni (KU Leuven), prof. dr. Wim Robberecht (KU Leuven), prof. dr. M. Cruts (UA), prof. dr. Stefanie Dedeurwaerdere (UA), prof. dr. Jan Gettemans (UGent), prof. dr. Geert van Loo (UGent), prof. dr. Ann Massie (VUB), prof. dr. F. Tissir (UCL), prof. dr. Etienne Olivier (UCL), prof. dr. Jean-Noël Octave (UCL), prof. dr. S.N. Schiffmann (ULB), prof. dr. P. Vanderhaeghen (ULB), prof. dr. P. Maquet (Ulg), prof. dr. L. Nguyen (ULg).

Op 15 mei vond de ceremonie plaats van de uitreiking van de onderzoekskredieten aan de verschillende teams en werden de beste onderzoeksrapporten van het vorige jaar bekroond met de volgende prijzen:
- De Prijs UCB werd toegekend aan prof. dr. Claudia Bagni (KU Leuven)
- De Prijs Burggravin Valine de Spoelberch werd toegekend aan dr. Fadel Tissir (UCL)
- De Prijs Solvay werd toegekend aan dr. Laurent Nguyen (ULg)
- De Prijs Janine et Jacques Delruelle werd toegekend aan prof. dr. Pierre Maquet (ULg)
- De Prijs van Gysel de Meise werd toegekend aan prof. dr. Vincent Timmerman (UA)

Het geplande laboratorium bezoek in de herfst werd uitgesteld tot 13 januari 2015, als gevolg van de zeer drukke agenda van Prinses Astrid.

Het wetenschappelijk comité dankt van harte de Raad van Bestuur voor hun vrijgevigheid ten voordele van het fundamenteel onderzoek in de neurowetenschappen en in het bijzonder Prinses Astrid, de Erevoorzitster, voor Haar voortdurende inzet en Haar interesse voor de vooruitgang van het onderzoek in de neurowetenschappen.
Moge Zij hier de uitdrukking vinden van onze oprechte waardering.

Prof. em. dr. Baron de Barsy,
wetenschappelijk directeur
Brussel, december 2014

Fondation Médicale Reine Elisabeth
2014
Introduction rapport d'activités de la FMRE - GSKE

L'année 2014 a été l'année de départ d'un nouveau cycle de trois ans (2014-15-16) pour les équipes de recherche sélectionnées.

Le conseil d'administration a confirmé le classement proposé par le comité scientifique et a accordé la somme de 40.000 euros par an pendant 3 ans, pour autant que la situation financière le permette, aux équipes suivantes : prof. dr. C. Bagni (KU Leuven), prof. dr. Wim Robberecht (KU Leuven), prof. dr. M. Cruts (UA), prof. dr. Stefanie Dedeurwaerdere (UA), prof. dr. Jan Gettemans (UGent), prof. dr. Geert van Loo (UGent), prof. dr. Ann Massie (VUB), prof. dr. F. Tissir (UCL), prof. dr. Etienne Olivier (UCL), prof. dr. Jean-Noël Octave (UCL), prof. dr. S.N. Schiffmann (ULB), prof. dr. P. Vanderhaeghen (ULB), prof. dr. P. Maquet (Ulg), prof. dr. L. Nguyen (ULg).

Le 15 mai a eu lieu la cérémonie de remise des enveloppes de crédit aux différentes équipes et la remise des prix, couronnant les meilleurs rapports de recherche de l'année précédente.
- Le Prix UCB a été attribué au prof. dr. Claudia Bagni (KU Leuven)
- Le Prix Vicomtesse Valine de Spoelberch a été attribué au dr. Fadel Tissir (UCL)
- Le Prix Solvay a été attribué au dr. Laurent Nguyen (ULg)
- Le Prix Janine et Jacques Delruelle a été attribué au prof. dr. Pierre Maquet (ULg)
- Le Prix van Gysel de Meise a été attribué au prof. dr. Vincent Timmerman (UA)

La visite de laboratoire prévue pour l'automne a été remise et postposée au 13 janvier 2015 en raison de l'agenda très chargé de la Princesse Astrid .

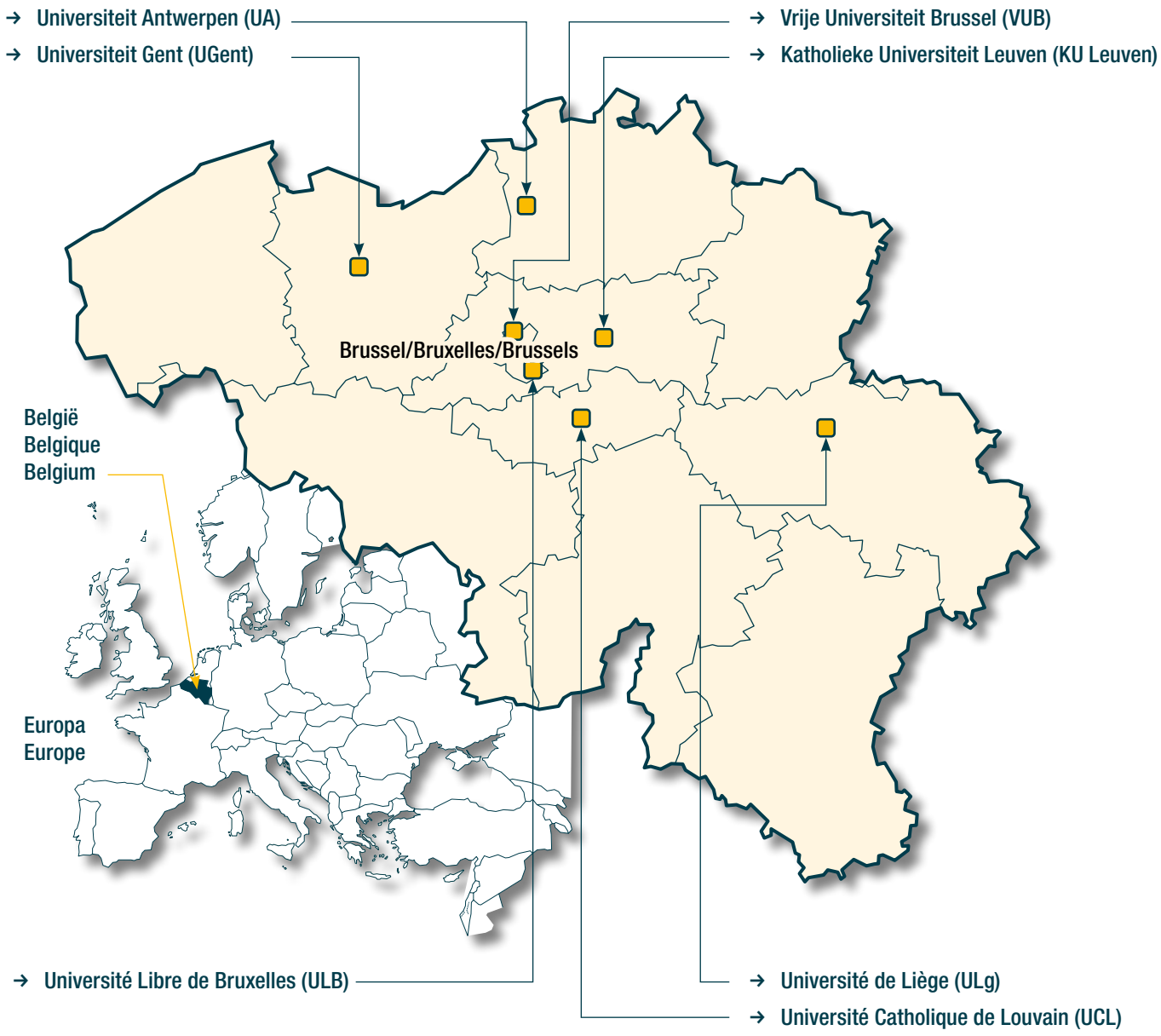
Le comité scientifique remercie chaleureusement le conseil d'administration de sa générosité en faveur la recherche fondamentale en neurosciences et tout particulièrement la Princesse Astrid, sa présidente d'honneur, pour son engagement permanent et son attention soutenue dans le suivi des progrès de la recherche en neurosciences.
Qu'elle trouve ici l'expression de notre très sincère reconnaissance.

Prof. em. dr. Baron de Barsy,
directeur scientifique
Bruxelles, 30 décembre 2014

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 2014-2016

Programmes de recherche subventionnés par la F.M.R.E. - Programme 2014-2016

Q.E.M.F. funded research projects - Program 2014-2016

KU Leuven

- **Prof. dr. Claudia Bagni**
  CYFIP1-pathies: shared pathways in intellectual disabilities and psychiatric disorders.

- **Prof. dr. Wim Robberecht**
  The ephrin axon repellent system in amyotrophic lateral sclerosis.

UA

- **Prof. dr. Marc Cruts, PhD & prof. Ilse Gijselinck**
  Integrative-Omics studies of frontotemporal lobar degeneration and related diseases.

- **Prof. dr. Stefanie Dedeurwaerdere, PhD**
  Translocator protein expression in temporal lobe epilepsy: picturing a Janus face?

UCL

- **Prof. dr. Etienne Olivier, PhD & dr. Alexandre Zénon**
  Parkinson's disease revisited: a new vision of basal ganglia functions in the context of the Parkinson's disease.

- **Prof. dr. Jean-Noël Octave**
  Alteration of cholesterol turnover in Alzheimer disease: molecular mechanisms and therapeutic applications.

- **Dr. Fadel Tissir**
  Shaping the nervous system: role of the planar cell polarity genes.

UGent

- **Prof. dr. Jan Gettemans, PhD**
  Offsetting gelsolin degradation in a transgene mouse model by means of chaperone nanobodies.

- **Prof. dr. Geert van Loo, PhD**
  Endoplasmic reticulum stress in autoimmune central nervous system inflammation and demyelination.

ULB

- **Prof. dr. Serge N. Schiffmann**
  Basal ganglia's functions and disorders: from specific genes and signalling pathways to neuronal sub-populations.

- **Dr. Pierre Vanderhaeghen, MD, PhD & dr. Anja Hasche**
  How to make cortical neurons: mechanisms, evolution and diseases.

ULg

- **Prof. dr. Pierre Maquet & dr. Christophe Phillips, ir**
  Decoding spontaneous mnemonic brain activity during post-training wakefulness and sleep using high-density EEG and electro-corticography.

- **Dr. Laurent Nguyen & dr. Brigitte Malgrange**
  Deciphering the role of protein acetylation in primary ciliogenesis.

VUB

- **Prof. dr. Ann Massie, PhD & prof. dr. Ilse Smolders**
  System Xc- as a potential target for novel neuroprotective strategies: focus on parkinson's disease and its psychiatric comorbidities.

Progress reports of the university research groups, supported by the Queen Elisabeth Medical Fondation in collaboration with the following professors and doctors (2014)

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

Progress report of the research group of

Prof. dr. Claudia Bagni

Katholieke Universiteit Leuven (KU Leuven)

Principal investigator

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CYFIP1-pathies: shared pathways in Intellectual Disabilities and Psychiatric Disorders

1. Research program summary

The human synapse proteome is disrupted by gene mutations causing over 100 brain diseases. These synaptopathies cause major psychiatric, neurological and childhood developmental disorders. Altered synaptic connectivity and plasticity are evident in schizophrenia (SCZ), autism spectrum disorder (ASD), intellectual disability (ID), major depressive disorder (MDD), Alzheimer disease (AD) as well as epilepsy (EPY), all disorders characterized by spine dysmorphogenesis.
One key molecule affecting synaptic structure and function is the Cytoplasmic FMRP Interacting Protein 1 (CYFIP1). At synapses CYFIP1 is involved in two distinct biological processes: with the protein causing the Fragile X Syndrome (FMRP), it modulates synaptic mRNA translation; as part of the WAVE regulatory complex, it is involved in actin cytoskeleton remodeling. We have recently shown, using a mouse model, that CYFIP1 shapes dendritic spines upon synaptic stimulation orchestrating these two molecular functions.
Deletions and duplications of the locus containing CYFIP1 have been identified in several neurological disorders. Furthermore, we have recently shown that reduction of CYFIP1 leads to spine dysmorphogenesis and that the CYFIP1 interactome is enriched in proteins associated to SCZ, ASD, ID and EPY. While those disorders are apparently heterogeneous, we believe that there are common defects in certain signaling pathways, and that CYFIP1 could be the “entry key” into a better understanding of these disorders. In this project we will undertake a “bed to bench and back” approach studying at cellular and molecular level patients with CYFIP1 mutations and patients with other synaptopathies. Finally we hope to use some of the identified pathways as possible biomarkers for those diseases and set the ground for appropriate pharmacological therapies for ASD, FXS, MDD, SCZ, AD.

2. Outcome

We and others have shown that the CYFIP1 gene has a critical role in synaptic structure and function. Furthermore, variants in the CYFIP1 gene have been associated to schizophrenia and ASD. How CYFIP1 deficiencies and or mutations contribute to those intellectual disabilities remains to be seen in human. In collaboration with colleagues at UZ Leuven, we identified a patient with a unique combination of CYFIP1 mutations. This clinical finding has a major impact on the field of human genetics, intellectual disabilities and Autism because, to our knowledge, this is the first clinical case identified with a point mutation in CYFIP1 and severe Autism. This clearly implicates CYFIP1 whereas the so-far identified BP1-BP2 deletion syndromes encompass 4 genes (including CYFIP1). In conclusion, during this first year of support we have deepened the current knowledge on CYFIP1 synaptic complex/es formation and functions in cells from patients with ASD and other synaptopathies through four aims. Importantly, because of the cellular and molecular studies we are able to perform on the patient’s cells we are confident that these generated findings will be published in a highly relevant scientific journal.

3. Achievements

Aim 1: *CYFIP1 and CYFIP1 interactome in patients with Intellectual Disabilities*.

Achievement 1: Human sample collection. During this first year of this grant support we gathered brain specimens from publicly available banks as the Stanley Medical Research Institute and the Maryland Brain Bank. Both provided us with protein extracts from different brain areas of patients with schizophrenia, bipolar disorder, FXS, ASD and unaffected controls. Furthermore, we have collected human skin primary cells (fibroblasts), lymphoblastoid cells and lymphocytes from patients with Autism Spectrum Disorders (ASDs) through our collaborators at UZ Leuven (Prof. Koenrad De Vrient and Prof. Hilde Peeters) and at Tor Vergata Hospital (Prof. Paolo Curatolo). **Figure 1**. Preliminary data on human cingulate cortex showed that two interactors of CYFIP1, namely FMRP and DCTN1, are downregulated in patients with Schizophrenia (n=14) and Bipolar Disorder (n=13). Postmortem tissue from cingulate cortex of patients with Bipolar Disorder (BD, n=13), Schizophrenia (SCZ, n=14) and unaffected controls (CTRL, n=15) were provided by the Stanley Medical Research Institute. FMRP and DCTN1 levels were analyzed blindly by Western blotting and normalized to Vinculin. *P-value<0.05. One-way ANOVA followed by post-hoc Bonferroni correction. These first dataset show that DCTN1 and FMRP are down-regulated in human postmortem brains of patients with BD and SCZ: two typical examples of synaptopathies.

Future plans 1: During the next two years of support we plan to further deepen the involvement of CYFIP1 and some of its protein partners (De Rubeis et al., Neuron 2013) in ASD and other disabilities as schizophrenia. We will analyze, by quantitative Western blotting, the different brain areas from which we have received samples so far together with additional ones that will be requested to the Autism Tissue Program brain banks (USA) for the following CYFIP1 interacting proteins: the Fragile X Mental Retardation Protein (FMRP), eukaryotic initiation factor (eIF4E), Dynactin (DCTN1), Caprin-1 (CAPR1), 24S-cholesterol hydroxylase (CYP46), Post-Synaptic Zip 45 (HOMER-1), Microtubule associated protein 2 (MAP2), Nck-associated protein 1 (NCKAP1), postsynaptic density protein 95 (PSD-95). The choice of these proteins among the 40 interactors we have identified (De Rubeis et al., 2013 Neuron) is based on the possible described association to SCZ, MDD, ID and ASD as well as their role as modulator of mRNA translation and actin remodeling, both processed highly affected in the above mentioned diseases.

Aim 2: *CYFIP1 interactome in the patient with the CYFIP1 R826Q mutation identified at UZL.*

Achievement 2: Characterization of a patient with severe ASD and a double mutation in the CYFIP1 gene. 15q11.2 copy number variations have been found in patients featuring autism, schizophrenia, neurodevelopmental delay and intellectual disability (ID). Although the size of the region 15q11.2 is still under discussion, four genes are widely accepted to be involved: Non-imprinted in Prader-Willi/Angelman syndrome (*NIPA) 1*, *NIPA2*, Cytoplasmic FMRP interacting protein 1 (*CYFIP1)* and Tubulin gamma complex associated protein 5 (*TUBGCP5)*. Due to its crucial function during synaptic development and neuronal connectivity, CYFIP1 is thought to contribute to the clinical phenotype observed in patients with 15q11.2 variations. In a collaborative work with Prof. Koenrad De Vrient and Prof. Hilde Peeters we identified and characterized a patient with severe ASD with a BP1 and BP2 deletion on chromosome 15q as well a point mutation on the other allele. Exome sequencing from the patient's DNA revealed a 15q11.2 deletion (15q11.2 del) and a single-nucleotide variation (SNV, p.R826Q) in *CYFIP1*. Sequence analysis revealed that the 15q11.2 del was inherited from the mother and the SNV from the father. Exome sequencing identified a small *de novo* deletion within chromosome 5q14.3 corresponding to exon 2 of the transcription factor *MEF2C* (Figure 3a), removing the start codon, i.e., this allele does not produce protein (data not shown). Because deletions within 5q14.3, including *MEF2C* mutations, have been associated with severe IDs we investigated the *MEF2c* expression level. Interestingly, the total *MEF2c protein* level is not different from control (Figure 3b) possibly due

to compensatory mechanisms. We could therefore exclude a contribution of MEF2C to the clinical phenotype of this patient. Based on the crystal structure of WRC, molecular modeling of the alteration introduced by the CYFIP1 R826Q suggested a loss of electrostatic interaction between CYFIP1 and three key components of its interactome namely NCKAP1, WAVE, ABI2 (Figure 3a).

Achievement 3. The CYFIP1 protein produced in patient's fibroblasts cannot be correctly assembled and interact with the macromolecular complex involved in actin polymerization. At first we set up the conditions to immunoprecipitate CYFIP1 from primary skin cells (fibroblasts) of unaffected individuals (n=7) and from the patient with the point mutation in CYFIP1 described above collected and expanded the seven control fibroblasts and the patient fibroblasts.
Upon transfection of YFP-CYFIP1 WT and YFP-CYFIP1 R826Q in HEK293T cells, we observed that the association of CYFIP1 with components of the WRC was reduced in cells transfected with mutated CYFIP1 (Figure 3b). CYFIP1 R826Q has a major impact on the direct interaction with ABI2 as well on the entire WRC suggesting that the SNV might compromise the stability and consequently the activity of the WRC affecting the downstream actin nucleating activity of the Arp2/3 complex. Increased protein synthesis and affected actin remodeling have been implicated in ASD. Because CYFIP1 links the two cellular processes at synapses (De Rubeis 2013), we investigated the dual contribution of 15q11.2 and R826Q in these pathways in the patient's cells. Importantly, patient's fibroblasts showed an increase by 30% of newly synthetized proteins (data not shown) consistent with a role of CYFIP1 in negatively regulating protein translation[9].

Achievement 4. Actin polymerization and cell movement is affected in patient's cell. Additionally, biochemical separation of filamentous (F) and globular (G) actin showed a reduced F/G actin ratio in patient's cells compared to control cells (Figure 4a). Such a decrease is also supported by a reduction in phalloidin intensity that specifically binds to F actin (Figure 4b). Patient's cells displayed as well a difference in the cell morphology, appearing more elongated (ratio major/minor axis, Figure 4b). These deficits are CYFIP1 dependent because the reintroduction of YFP-CYFIP1 in patient's cells reverted these cellular phenotypes (Figure 4c). These results unequivocally implicated CYFIP1 dysfunction in the observed cellular defects. To finally demonstrate that actin polymerization and cell movement were affected, we monitored the expression of Lifeact-GFP in control and patient's fibroblasts. Patient's cells showed a clear reduction in protrusions number and length (Figure 4d). These results are consistent with a reduced migration and a higher cellular adhesion (data not shown). It is known that the CYFIP1-ABI2-WRC is active at the plasma membrane (Chen et al. Nature 2010). According to the CYFIP1 structure (Chen et al), modeled in Figure 3a, the SNV should promote a conformational change that stabilizes CYFIP1 at the plasma membrane.

Achievement 5. CYFIP1 R826Q confers stronger anchoring of CYFIP1 at the plasma membrane. We asked if the R826Q mutation has an effect on membrane anchoring. Membrane preparations from patient's cells confirmed such a prediction (Figure 4e). Although the patient has a BP1-BP2 deletion/R826Q mutation that ultimately leads to a decrease level of CYFIP1, the total amount of CYFIP1 at the membrane is the same as in control cells.
Such an imbalance between cytosolic and membrane-associated CYFIP1 may lead to deregulated signals towards the ARP2/3 complex, affecting actin cytoskeleton as shown in Figures 4a-b.

Future plans 2: We are scaling up our protocol for CYFIP1 immunoprecipitation to perform mass spectrometry analysis of the CYFIP1-mutant interacting proteins. We have already established a collaboration with Prof. Kris Gevaert, Leader of the VIB Proteomics Expertise Center in Gent to proceed with the proteomic analysis of the immunoprecipitated CYFIP1 interactome from patient's cells.

Aim 3: *Identification of the CYFIP1 mRNA repertoire from the patient with BP1-BP2 deletion and point mutation in CYFIP1.*

Future plans 3. Ad discussed in Aim 2, we have now established the protocol to efficiently immunoprecipitate CYFIP1 from human samples. The analysis of associated RNA will be performed in this coming year.

Aim 4: *Validation of the dysregulated identified molecules (putative biomarkers) through data base analysis and biospecimens examination.*

Achievement 6: CYFIP1 and its interactor eIF4E proteins are altered in patients with ASD. Whole blood from patients affected by non syndromic ASD with an age of 8-12 (n=7) and control (n=1) have been collected, mononuclear cells were separated by Histopaque-1077 (SIGMA). Total proteins were extracted from the cellular pellet and analyzed by Western blotting. From these preliminary data we observe a reduction of CYFIP1 and an increase in eIF4E. Of note, a mouse overexpressing eIF4E has been shown to exhibit ASD feature (Santini et al., 2012. Nature).

Future plans 4: This analysis will be expanded to a larger number of samples and will include in addition to CYFIP1 and eIF4E: the Fragile X Mental Retardation Protein (FMRP), Dynactin (DCTN1), Caprin-1 (CAPR1), 24S-cholesterol hydroxylase (CYP46), Post Synaptic Zip 45 (HOMER-1), Microtubule associated protein 2 (MAP2), Nck-associated protein 1 (NCKAP1). We aim at identifying a molecular signature for certain forms of ASD that can be monitored in the future directly from blood samples.

4. Networking and collaborations

The project results from the integration of complementary expertise, giving us the opportunity to create productive scientific collaborations in our Institute and with other institutions abroad. First, the mass spectrometry analysis is currently conducted in collaboration with a group with a long-lasting experience in mass spectrometry techniques, namely the group of Prof. Kris Gevaert (University of Gent), leader of the VIB Proteomics Expertise Center. Furthermore, we are actively collaborating with clinical and human geneticists at UZ Leuven (Prof. Koenrad De Vrient and Prof. Hilde Peeters) and at Tor Vergata Hospital (Prof. Paolo Curatolo). Finally, we have a fruitful collaboration with the microscope imaging facility of the Center of Human Genetics, KU Leuven (Light Microscopy & Imaging Network, LiMoNe) for spine visualization and analysis.

5. Relevance

The work we have performed with the support of the FMRE helped us to increase the current knowledge of CYFIP1 distribution and function/s in brain first using a mouse model and, importantly, during this last year through the identification of the first patient with a double hit mutation in the CYFIP1 gene. We aim at understanding how a single molecule, CYFIP1, affecting two key cellular processes as protein synthesis and actin remodeling if mutated or reduced causes ASD. We think to have in our hands the key to unravel such a mysterious disease as ASD. The complete proteomic and transcriptomic of the identified patient's cells will complement the cellular and molecular studies we have performed so far that, due to the relevance for ASD and possibly other synaptopathies, will be submitted for publication to Nature Genetics in the coming weeks. We hope that the *Fondation Médical Reine Elisabeth* will continue to support our research.

6. Publications in preparation based on the FMRE support (2014-2016)

– Ana Rita Santos, Veerle De Wolf, Esperanza Fernandez, Daniele Di Marino, Tilmann Achsel, Sebastian Dupraz, Frank Bradke, Hilde Van Esch, Koenraad Devriendt, Hilde Peeters and Claudia Bagni (2015). "CYFIP1 dysfunctions in protein synthesis and actin remodeling in a severe case of ASD"**.** *To be submitted to Nature Genetics next month.*

7. Team key publications (5 selected from 25 publications over the last five years)

– La Fata G, Gärtner A, Domínguez-Iturza N, Dresselaers T, Dawitz J, Poorthuis RB, Averna M, Himmelreich U, Meredith RM, Achsel T, Dotti CG and **Bagni C** (2014). "The Fragile X Mental Retardation Protein regulates neuronal multipolar-bipolar transition and affects cortical circuitry in the developing cortex" Nature Neurosci., doi: 10.1038/nn.3870. Epub ahead of print.
– Pasciuto E and **Bagni C** (2014). "FMRP mRNA Targets and Associated Human Diseases" Cell *SnapShot*, 158 (6): 1446-1446.
– Pasciuto E and **Bagni C** (2014). "FMRP Interacting Proteins" Cell *SnapShot* 159 (1):218-218.
– De Rubeis S, Pasciuto W, Li Ka Wan, Fernández E, Di Marino D, Buzzi A, Ostroff L, Klann E, Zwartkruis F, Komiyama NH, Grant S, Choquet D, Poujol C, Achsel T, Posthuma D, Smit AB and **Bagni C** (2013). CYFIP1 coordinates mRNA translation and cytoskeleton remodeling to ensure proper dendritic spine formation. Neuron, 79: 1169-1182.
– Luca R*, Averna M*, Vecchi M*, Zalfa F*, La Fata G, del Nonno F, Nardacci R, Bianchi F, Bianchi M, Nuciforo P, Munck S, Parrella P, Moura R, Signori E, Alston R, Farace MG, Fazio VM, Piacentini M, De Strooper B, Neri G, Neven P, Evans DG, Carmeliet P, Mazzone M and **Bagni C** (2013). ***The Fragile X Protein binds mRNAs involved in cancer progression and modulates metastasis formation***. ***EMBO Molecular Medicine*** 5: 1523-1536.

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Progress report of the research group of

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Integrative -Omics Studies of Frontotemporal Lobar Degeneration and Related Diseases

1. Specific Aims

Frontotemporal dementia (FTD), semantic dementia (SD), and progressive nonfluent aphasia (PNFA) are disabling and irreversible clinical conditions that are characterized by progressive neuronal loss in the frontal and/or temporal cortices, collectively referred to as frontotemporal lobar degeneration (FTLD) (see Sieben et al., 2012 for review). Onset age of FTLD ranges on average from 45 to 65 years and often affects people who are mid-career and raising a family. In this age group, FTLD is the second most common type of neurodegenerative dementia after Alzheimer's disease (AD). Due to the aging population in Belgium and worldwide, the incidence of FTLD will exponentially increase in the years to come. Yet, there are no preventive or curative treatments available for FTLD today, although symptomatic treatments are frequently used to manage behavioral abnormalities associated with this disorder (Boxer and Boeve, 2007). In order to develop effective therapies aiming at delaying, halting or possibly preventing the disease, our understanding of the pathological mechanisms leading to the neurodegenerative processes in the patients' brains is essential.

In up to 50% of patients, familial aggregation has been observed suggesting highly penetrant genetic factors. Mutations in three genes (*MAPT*, *GRN*, *C9orf72*) were identified as a frequent cause of FTLD (FTLD Mutation Database, Cruts et al., 2012), together explaining about 20 to 50% of familial FTLD. In addition, rare mutations were reported in *VCP*, *TARDBP*, *FUS*, and *CHMP2B*. Together, these genes provide limited information on the pathomechanisms of FTLD. To this end, and to improve differential diagnostic efficacy, the identification of additional disease genes causing or modifying the expression of the FTLD symptoms is essential. Therefore, we aim to further expand our understanding of the gene networks and biological processes that are affected in FTLD. Further, onset age in FTLD is typically highly variable, suggesting that genetic factors modify the onset age and severity of the clinical symptoms of the disease. We hypothesize that genes modifying onset age are excellent therapeutic targets to delay and/or prevent disease onset and progression. Therefore, we will identify disease-modifying genes.

As a direct result of these studies, improved early diagnosis can be offered to the patients and their families. Further we anticipate that results obtained in this project will be the basis for subsequent cell biology studies of the functions and dysfunctions of pathways contributing to neurodegeneration in FTLD. These pathways might be involved in related neurodegenerative diseases, as we have demonstrated e.g. for the FTLD gene *C9orf72*, which is also contributing to ALS (Gijselinck et al., 2012) and AD (Cacace et al., 2013), and *GRN*, which is also contributing to AD (Brouwers et al., 2007; Brouwers et al., 2008), Parkinson's disease (Brouwers et al., 2007) and ALS (Sleegers et al., 2008).

In this perspective, the specific objectives set out in this project were:
1. Unraveling the disease mechanisms associated with *C9orf72* repeat expansion
2. Identification of novel causal genes for FTLD
3. Identification of genes modifying onset age in FTLD

Progress was made in each of these objectives as detailed below.

2. Disease mechanisms associated with *C9orf72* repeat expansion

In the Q.E.M.F. granting period 2011-2013, we identified a pathological repeat expansion in the proximal regulatory region of *C9orf72* as a very frequent cause of disease in the FTLD/ALS spectrum (Gijselinck et al., 2010; Gijselinck et al., 2012). Because diverse disease mechanisms have been associated with pathological repeat expansions, we will studied the incompletely understood mechanism by which the *C9orf72* repeat expansion causes disease (Cruts et al., 2013).

We investigated the involvement of a toxic gain-of-function mechanism by studying the effect of repeat expansion length on onset age using Southern blot analysis in related and unrelated *C9orf72* expansion carriers and the role of a loss-of-function mechanism by reporter gene expression and methylation studies of the *C9orf72* promoter. Repeat expansion sizes in blood ranged from 45 to over 2100 G_4C_2 units. We detected short expansions (45-78 units) in 6.5% of carriers and demonstrated segregation of a repeat of 50 units in an FTLD family, indicating that expansions as short as 50 units may cause FTLD. Also, we showed for the first time negative correlation between repeat expansion size and onset age ($p=0.0006$) (**Figure 1**). Further, an increment of 1500 units was observed from parent to offspring in one family, pointing to genetic anticipation. We established a gradually decreasing *C9orf72* promoter activity with an increasing size of intermediate G_4C_2 repeats, and a decreasing transcriptional activity of small deletions in the 3' flanking low complexity sequence in human kidney and neuroblastoma cells ($p<0.0001$). Further, we observed increased DNA methylation of G_4C_2 intermediate repeats and the 5' flanking CpG island in carriers of intermediate and expanded repeats ($p<0.0001$). We provided evidence for both gain and loss-of-function disease mechanisms possibly acting on different transcript variants to be involved in the disease process (Gijselinck et al., 2014).

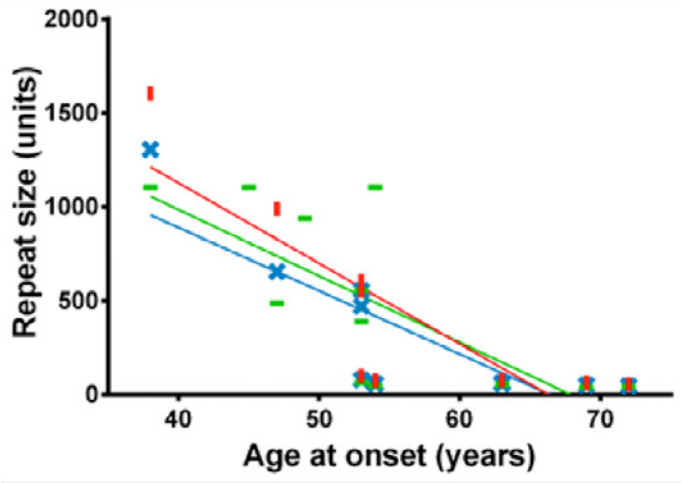

Figure 1. Correlation of *C9orf72* repeat expansion size with disease onset age. Correlation plot between age at onset and repeat expansion size for maximum (red), minimum (green) and median (blue) repeat length as determined in 13 repeat expansion patients by Southern blot hybridization and small repeat expansion PCR. Trend lines are indicated in corresponding colors (Gijselinck et al., 2014).

3. Novel causal genes for FTLD

3.1. *C9orf72*

We identified a pathological repeat expansion in *C9orf72* as a very frequent cause of FTLD and ALS (Gijselinck et al., 2010; Gijselinck et al., 2012). In the study we report now, we aimed to clarify the role of $(G_4C_2)_n$ expansions in the etiology of Parkinson disease (PD) in the worldwide multicenter Genetic Epidemiology of Parkinson's Disease (GEO-PD) cohort. *C9orf72* $(G_4C_2)_n$ repeats were assessed in a GEO-PD cohort of 7,494 patients diagnosed with PD and 5,886 neurologically healthy control individuals ascertained in Europe, Asia, North America, and Australia. A pathogenic $(G_4C_2)_{n>60}$ expansion was detected in only 4 patients with PD (4/7,232; 0.055%), all with a positive family history of neurodegenerative dementia, amyotrophic lateral sclerosis, or atypical parkinsonism, while no carriers

were detected with typical sporadic or familial PD. Meta-analysis revealed a small increase in risk of PD with an increasing number of G_4C_2 repeat units; however, we could not detect a robust association between the *C9orf72* $(G_4C_2)_n$ repeat and PD, and the population attributable risk was low. Together, these findings indicate that expansions in *C9orf72* do not have a major role in the pathogenesis of PD. Testing for *C9orf72* repeat expansions should only be considered in patients with PD who have overt symptoms of frontotemporal lobar degeneration/amyotrophic lateral sclerosis or apparent family history of neurodegenerative dementia or motor neuron disease (Theuns et al., 2014).

3.2. *TREM2*

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 *APOE* ε4. We systematically screened the *TREM2* coding region within a Belgian study on neurodegenerative brain diseases comprising 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 approximately 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 = 2.93x10-17). 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).

3.3. *VPS13C*

In about 60% of the families with FTLD, the genetic cause is still unknown. Therefore, we performed whole genome sequencing on 16 unrelated familial FTLD patients to identify rare, highly penetrant mutations. Annotation and analysis of the genome sequences was performed using GenomeComb and variants were filtered and prioritized using multiple genetic and functional criteria. Analysis of these variants in 392 Belgian FTLD patients and 900 control individuals revealed the presence of two novel coding missense variants (p.W395C and p.A444P) in the vacuolar protein sorting 13 homolog C gene (*VPS13C*). The p.A444P missense mutation was present in a WGS patient and two additional index patients. The p.W395C missense mutation was present in an affected sib pair who was also carrying the p.A444P mutation. Screening of the 86 coding exons of *VPS13C* in the FTLD population (n = 590) was performed using the Multiplex Amplification of Specific Targets for Resequencing (MASTR) technology and resulted in the identification of 19 additional missense mutations that were absent in at least 1314 age- and geographically matched control individuals, resulting in an overall mutation frequency of 4.2% (25 of 590 FTLD patients). Immunocytochemistry of lymphoblast cells of five patients with a *VPS13C* mutation suggested a 44-80% decreased expression (p<0.001) of VPS13C protein compared to mutation-negative controls (n=4). Further neuropathological and cell biological studies are needed to support the role of *VPS13C* in FTLD (Philtjens et al., 2014).

4. Onset age modifying genes for FTLD

4.1. A major onset age modifier locus on chromosome 12

In FTLD a wide distribution of onset age, ranging from 20 to 91 years, suggests a significant contribution of factors modifying the disease onset. In a Flanders-Belgian FTLD founder pedigree segregating a *GRN* null mutation and exhibiting a wide onset age range between 45 and 84 years, we excluded an effect on onset age of functional candidate modifiers such as the unaffected copy of *GRN* and

variations in *TMEM106B*. In the family we identified a QTL for onset age with strong evidence of linkage on chromosome 12 (Bayes' factor > 10) (Shugart, 2014). This locus of 7 Mb contains 119 genes and explains up to 91% of the genetic variance in onset age. A set of 122 candidate modifier variations was selected from whole genome sequencing data of 23 mutation carriers. All variations associated with onset age with a p-value < 0.01 (n=19) are located in intronic or intergenic regions throughout the priority region of the QTL. To identify the functional variation, *in silico* clues are being sought, as well as biological evidence in brain transcriptome and serum proteome data of the family and in targeted expression analyses of candidate modifier genes, by comparing expression levels between patients with early and late onset of disease. Candidate modifier variations and genes will be studied in extended patient cohorts. The identification of genetic modifiers may shed further light on the disease mechanisms of FTLD and may provide stepping stones for the development of therapies that can halt or delay, or even prevent the disease. (Wauters et al., 2014).

4.2. *TMEM106B*

Genetic variants at *TMEM106B* influence risk for the most common neuropathological subtype of FTLD, characterized by inclusions of TAR DNA-binding protein of 43 kDa (FTLD-TDP). Previous reports have shown that *TMEM106B* is a genetic modifier of FTLD-TDP caused by progranulin (GRN) mutations, with the major (risk) allele of rs1990622 associating with earlier age at onset of disease (Van Deerlin et al., 2010). Now, we reported that the rs1990622 genotype affects age at death in a single-site discovery cohort of FTLD patients with *C9orf72* repeat expansions (n = 14), with the major allele correlated with later age at death (p = 0.024). We replicated this modifier effect in a 30-site international neuropathological cohort of FTLD-TDP patients with *C9orf72* expansions (n = 75), again finding that the major allele associates with later age at death (p = 0.016), as well as later age at onset (p = 0.019). In contrast, the *TMEM106B* genotype does not affect age at onset or death in 241 FTLD-TDP cases negative for *GRN* mutations or *C9orf72* expansions. Thus, *TMEM106B* is a genetic modifier of FTLD with *C9orf72* expansions. Intriguingly, the genotype that confers increased risk for developing FTLD-TDP (major, or T, allele of rs1990622) is associated with later age at onset and death in *C9orf72* expansion carriers, providing an example of sign epistasis in human neurodegenerative disease (Gallagher et al., 2014).

4.3. Genome-wide association study

In a large international study, we sought to identify novel genetic risk loci associated with the FTD. We did a two-stage genome-wide association study on clinical FTD, analyzing samples from 3526 patients with FTD and 9402 healthy controls. To reduce genetic heterogeneity, all participants were of European ancestry. In the discovery phase (samples from 2154 patients with FTD and 4308 controls), we did separate association analyses for each FTD subtype (behavioral variant FTD, semantic dementia, progressive non-fluent aphasia, and FTD overlapping with motor neuron disease i.e. FTD-MND), followed by a meta-analysis of the entire dataset. We carried forward replication of the novel suggestive loci in an independent sample series comprising 1372 patients and 5094 controls, and then did joint phase and brain expression and methylation quantitative trait loci (QTL) analyses for the associated ($p < 5 \times 10^{-8}$) single-nucleotide polymorphisms. We identified novel associations exceeding the genome-wide significance threshold of $p < 5 \times 10^{-8}$). Combined (joint) analyses of discovery and replication phases showed genome-wide significant association at 6p21.3, HLA locus (immune system), for rs9268877 ($p = 1.05 \times 10^{-8}$; odds ratio=1.204 [95% CI 1.11-1.30]), rs9268856 ($p = 5.51 \times 10^{-9}$; 0.809 [0.76-0.86]) and rs1980493 ($p = 1.57 \times 10^{-8}$, 0.775 [0.69-0.86]) in the entire cohort. We also identified a potential novel locus at 11q14, encompassing *RAB38/CTSC*, the transcripts of which are related to lysosomal biology, for the behavioral FTD subtype for which joint analyses showed suggestive association for rs302668 ($p = 2.44 \times 10^{-7}$; 0.814 [0.71-0.92]). Analysis of expression and methylation QTL data suggested that these loci might affect expression and methylation in cis. These findings suggest that immune system processes (link to 6p21.3) and possibly lysosomal and autophagy pathways (link to 11q14) are potentially involved in FTD. Our findings need to be replicated to better define the association of the newly identified

loci with disease and to shed light on the pathomechanisms contributing to FTD (Ferrari et al., 2014). These novel loci may provide insight into genes and or pathways harboring genetic variability modifying onset age in FTLD.

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6.2. Meeting abstracts in international journals

- Philtjens,S., **Gijselinck,I.**, Van Langenhove,T., van der Zee,T., Engelborghs,S., Vandenbulcke,M., Vandenberghe,R., Santens,P., De Deyn,P.P., Van Broeckhoven,C., **Cruts,M.**, BELNEU consortium: Next generation sequencing identifies mutations in VPS13C associated with decreased expression of endogenous protein in Frontotemporal lobar degeneration. Am J Neurodegener Dis 3 Suppl 1: 13 (2014)
- Wauters,E., **Gijselinck,I.**, Van Langenhove,T., Engelborghs,S., Vandenbulcke,M., Mattheijssens,M., Peeters,K., Martin,J.-J., Cras,P., Santens,P., Vandenberghe,R., De Deyn,P.P., van der Zee,J., Sleegers,K., Van Broeckhoven,C., **Cruts,M.**: An integrative approach to identify onset age modifier genes in a large founder GRN FTLD family. Am J Neurodegener Dis 3 Suppl 1: 271 (2014)
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- **Cruts,M.**, **Gijselinck,I.**, Philtjens,S., van der Zee,J., Maes,G., Engelborghs,S., Vandenbulcke,M., Vandenberghe,R., Santens,P., De Deyn,P.P., Van Broeckhoven,C., BELNEU consortium: Genome-wide screen in FTLD/ALS patient cohorts for pathological G4C2 repeat expansions other than C9orf72. Am J Neurodegener Dis 3 Suppl 1: 280 (2014)
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6.3. Abstracts in abstract books of international meetings

- Philtjens,S., **Gijselinck,I.**, Van Langenhove,T., van der Zee,T., Engelborghs,S., Vandenbulcke,M., Vandenberghe,R., Santens,P., De Deyn,P.P., Van Broeckhoven,C., **Cruts,M.**: Whole genome sequencing identifies vps13c as a candidate gene for frontotemporal lobar degeneration. Human Genome Meeting, Geneva Switzerland: OR014 (April 27-30, 2014)
- **Gijselinck,I.**, Van Langenhove,T., Philtjens,S., van der Zee,J., Engelborghs,S., Sieben,A., De Jonghe,P., Vandenberghe,R., Santens,P., De Bleecker,J., Robberecht,W., Cras,P., De Deyn,P.P., Van Broeckhoven,C., **Cruts,M.**: C9orf72 G4C2 repeat expansion size associates with genetic anticipation in FTLD and ALS. Human Genome Meeting, Geneva Switzerland: OR014 (April 27-30, 2014)

6.4. Honors, Prizes & Awards

Travel Awards

- Gijselinck I.: Funding of the FWO for participating in a conference abroad: 9th International Conference on Frontotemporal dementias, Vancouver, Canada, October 23-25, 2014
- Wauters E.: Student bursary, complimentary conference registration and accommodation, supported by the International Society for Frontotemporal Dementias to attend the 9th International Conference on Frontotemporal Dementias, Vancouver, Canada, October 23-25, 2014

6.5. Presentations

Invited lectures

International

Cruts M.: *VPS13C* mutations associate with frontotemporal lobar degeneration and decreased protein expression. The Third Joint Symposium on Neuroacanthocytosis and Neurodegeneration With Brain Iron Accumulation. Stresa, Italy, October 30 – November 1 2014

Oral presentations

International

- Philtjens,S: Whole genome sequencing identifies vps13c as a candidate gene for frontotemporal lobar degeneration. Human Genome Meeting 2014. Geneva, Switzerland, April 27-30 2014
- Philtjens S.: Next generation sequencing identifies mutations in VPS13C associated with decreased expression of endogenous protein in Frontotemporal lobar degeneration. 9th International Conference on Frontotemporal Dementias. Vancouver, Canada, October 23 – 25 2014

National

- Wauters E.: Identification of onset age modifier genes in frontotemporal lobar degeneration: an integrative family-based strategy. VIB Seminar 2014. Blankenberge, Belgium, April 28-30, 2014

Poster presentations

International

- Gijselinck,I.: C9orf72 G4C2 repeat expansion size associates with genetic anticipation in FTLD and ALS. Human Genome Meeting 2014. Geneva, Switzerland, April 27-30 2014
- Wauters,E.: An integrative approach to identify onset age modifier genes in a large founder GRN FTLD family. 9th International Conference on Frontotemporal Dementias. Vancouver, Canada, October 23 – 25 2014
- Gijselinck,I.: C9orf72 G4C2 repeat size associates with genetic anticipation, hyper-methylation and transcriptional down-regulation in FTLD and ALS. 9th International Conference on Frontotemporal Dementias. Vancouver, Canada, October 23 – 25 2014
- Cruts,M.: Genome-wide screen in FTLD/ALS patient cohorts for pathological G4C2 repeat expansions other than C9orf72. . 9th International Conference on Frontotemporal Dementias. Vancouver, Canada, October 23 – 25 2014

National

- Wauters E.: An integrative approach to identify onset age modifier genes in a large founder GRN FTLD family. Annual Scientific IAP Meeting. Antwerp, Belgium, October 3, 2014

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

Progress report of the research group of

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Table of contents

Translocator protein expression in temporal lobe epilepsy: picturing a Janus face?

1. Scientific report of the progress made in 2014

Epilepsy is a chronic neurological condition characterized by recurrent seizures that affects about 65 millions of people worldwide. Epilepsy has a severe effect not only on the individual, but also on society since the estimated total cost of epilepsy in Europe in 2004 was over €15.5 billion. Temporal lobe epilepsy (TLE) is the most common and severe form of focal acquired epilepsy in humans and it is associated with psychiatric comorbidities such as anxiety and depression. Current drugs available are purely symptomatic, have many side effects, and in addition up to 40% of epilepsy patients still remain resistant to anti-epileptic drugs. Epileptogenesis is a dynamic disease process starting before the first symptoms/seizures occur. However, the neurobiological processes that result in acquired epilepsy still remain unclear, impeding the development of more potent, targeted and disease-modifying treatments.

1.1. Specific Aims

To fully exploit the potential of the translocator protein (TSPO) as an anti-inflammatory target or a biomarker for epilepsy on the long term, the objective of this proposal is to tease out the complexities of TSPO upregulation in a model of TLE.

The specific aims to meet this objective are:
- (A1) to describe the spatial and temporal profile of TSPO expression during epileptogenesis starting from the initiating insult until established epilepsy;
- (A2) to evaluate TSPO expression in multiple brain cell types during disease ontogenesis;
- (A3) to study the effect of TSPO on brain inflammation and steroid synthesis;
- (A4) to unravel the role of TSPO in the development of chronic epilepsy.

We have made significant progress regarding the first two aims in 2014, which resulted in the submission of a research paper to Neuropathology and Applied Neurobiology (ranked 7/76 in the category Pathology). In addition, we have initiated several studies to address the remaining aims.

1.2. Brain inflammation in a chronic epilepsy model: characterising the spatiotemporal evolution of the translocator protein using *post-mortem* and *in vivo* techniques (Amhaoul et al., *under revision in Neuropathology and Applied Neurobiology*)

A hallmark in the neuropathology of TLE is brain inflammation which has been suggested as both a biomarker and new mechanism for treatments. TSPO, due to its high upregulation under neuroinflammatory conditions and the availability of selective Positron Emission Tomography (PET) tracers, is a candidate target. An important step to exploit this target is a thorough characterisation of the spatiotemporal profile of TSPO during epileptogenesis.
We found that TSPO expression was dynamically upregulated during epileptogenesis and persisted in the chronic phase. TSPO expression correlated with microglia activation rather than reactive astrocytes. This suggests that microglia is the primary brain cell population responsible for TSPO over-expression. In the chronic phase, a clear relationship with seizures was lacking, therefore, TSPO upregulation does not seem a direct underlying mechanism for seizure generation. TSPO PET imaging provides a unique opportunity to non-invasively investigate brain inflammation during epileptogenesis within the same subject (Figure 1).

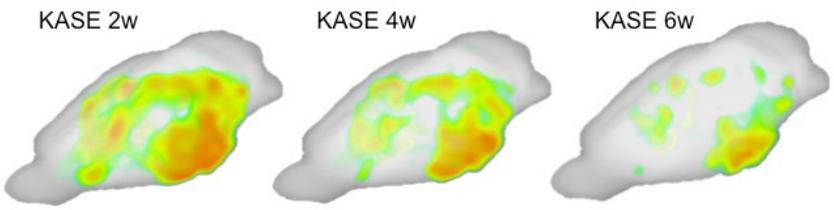

Figure 1: Serial 3-D ^{18}F-PBR111 PET images of a representative kainic acid-induced *status epilepticus* (KASE) rat merged with its MRI showing brain networks with increased brain inflammation. TSPO binding is peaking around 2 weeks post *status epilepticus,* a turning point when the first spontaneous seizures start to arise. Afterwards it is gradually declining, but remains elevated throughout the chronic epilepsy phase.

1.3. Imaging brain inflammation in epilepsy (Amhaoul et al., 2014)

Non-invasive imaging of brain inflammation can promote our fundamental knowledge, which may lead to better insights into the role of brain inflammation in disease ontogenesis. Moreover, it will allow us to investigate whether the visualization of this process can serve as a validated biomarker for epilepsy. In turn, such can lead to major perspectives regarding the development and evaluation of anti-inflammatory treatments, and screening possibilities for patients at risk. Here, we first discuss the applications for imaging of the different brain inflammation constituents. Secondly, we review the available approaches for molecular imaging of brain inflammation in general and finally present the current research on the imaging of brain inflammation in patients and experimental models of epilepsy. The current imaging toolbox is limited by the range of neuroinflammatory targets, which can be visualized at present, and in addition, the often indirect approaches used. We believe that research in this field will further advance as highly specific ligands, and producible and practical imaging approaches will become available.

1.4. Preliminary data

Post-traumatic epilepsy often occurs following a latent period of months to years as a consequence of a brain insult. Although this latent period clearly represents a therapeutic window, we have not been able to stratify patients at risk for post-traumatic epilepsy. Brain inflammation has been recognized as an important factor in the pathology of various types of epilepsy. The aim of an ongoing study is to determine network alterations associated with the process of epileptogenesis by means of TSPO PET scans, a biomarker of neuroinflammation, in the KASE model. Comparing TSPO PET scans using network analysis approaches showed promise for differentiating KASE animals that developed epilepsy from those who did not and controls. However, the limited number of KASE rats without seizures in the chronic epilepsy period warrants caution when interpreting the results. More animals will be studied to validate whether these approaches could be useful in identifying subjects who will develop epilepsy in the future.

2. New opportunities

Several opportunities have risen in the past year. Firstly, our preliminary PET data described above are very exciting and may reveal mechanisms involved in maladaptive plasticity during epileptogenesis. Therefore, we are planning to extend this study in 2015 and perform additional longitudinal measurements with more animals. This will allow us to characterize the evolution profile during epileptogenesis in the same animals, which is only possible through a longitudinal study with this state-of-the-art PET imaging technique.

Finally, since KASE animals without seizures seem to have a higher expression of TSPO than KASE animals with seizures, we will verify this possible protective function of TSPO in preventing seizures. Just very recently, several papers occurred describing a TSPO KO mice. We are in contact with one of these groups, to be able to use this new mice line to discern the effect of TSPO on brain inflammation and to evaluate the role of TSPO during disease ontogenesis of epilepsy.

3. Budget

As outlined in the description of the project proposal, the budget received from the GSKE was amended for the salary of laboratory assistant Mrs Van Eetveldt and to support animal purchase and housing, PET tracer and scanning costs, and various consumables (kainic acid, emapunil, ARG ligands, histology costs, etc). The next years, the awarded funding will be continued to be used for these purposes which is mandatory to sustain our research.

4. Scientific activities

4.1. Publications acknowledging G.S.K.E support:

- Pitkänen A, Ndode-Ekane XE, Lukasiuk K, Wilczynski GM, Dityatev A, Walker MC, Chabrol E, **Dedeurwaerdere S**, Vazquez N, Powell EM. ***Neural ECM and epilepsy.*** Prog Brain Res. 2014;214:229-62.
- **Amhaoul H**, Staelens S, **Dedeurwaerdere S**. ***Imaging brain inflammation in epilepsy.*** Neuroscience. 2014 Oct 24;279:238-52.
- **Amhaoul H**, Hamaide J, Reichel SN, Verhaeghe J, Geerts E, Van Dam D, De Deyn PP, Kumar-Singh S, Katsifis A, Van Der Linden A, Staelens S, **Dedeurwaerdere S**. Brain inflammation in a chronic epilepsy model: characterising the spatiotemporal evolution of the translocator protein using *post-mortem* and *in vivo* techniques. Submitted to Neuropathology and Applied Neurobiology (*under review*).

4.2. Presentations at national and international scientific meetings

Invited Lectures

Stefanie Dedeurwaerdere

29/06-3/7/2014	**11th European Congress on Epileptology:** *PET imaging in models of epileptogenesis and chronic epilepsy.*	Stockholm, Sweden

Poster presentations (presenting author)

Stefanie Dedeurwaerdere

21-24/05/2014	**NRM2014:** *Follow-up of early brain inflammation in a rodent model of epileptogenesis with post-mortem and in vivo imaging techniques.*	Amsterdam, The Netherlands
17-22/08/2014	**Gordon Research Conference on Mechanisms of Epilepsy & Neuronal Synchronization:** *Translocator protein as a neuroimaging biomarker of epileptogenesis in a rodent model of temporal lobe epilepsy.*	West Dover, USA

Halima Amhaoul

24-25/04/2014	**Imaging Neuroinflammation in Neurodegenerative Diseases (INMiND) TSPO Symposium 2014:** *Brain inflammation and epileptogenesis: a role for TSPO?*	Manchester, United Kingdom
4-6/06/2014	**European Molecular Imaging Meeting (EMIM) 2014:** *Brain inflammation in a chronic epilepsy model: determining the spatiotemporal profile of glial activation by in vivo 18F-PBR111 PET and standard immunohistochemistry techniques*	Antwerp, Belgium

Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
Königin-Elisabeth-Stiftung für Medizin
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Progress report of the research group of

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- prof. W. Derave (Department of Movement and Sport Sciences, Ghent University)
- prof. C. Cuvelier (Pathology dept. Ghent University)
- prof. T. Lahoutte (In Vivo Cellular and Molecular Imaging Laboratory, Free University Brussels)

New collaboration: prof. Marinee Chuah and prof. Thierry Vandendriessche (Department of Gene Therapy & Regenerative Medicine, Free University Brussels)

Offsetting gelsolin degradation in a transgene mouse model by means of chaperone nanobodies.

Background of the research proposal

1. Brief clinical introduction

Patients with *gelsolin amyloidosis* (FAF) diplay diverse clinical symptoms including early aging, periferal neuropathies affecting the cranial nerves, bilateral progressive facial paralysis, and corneal lattice dystrophy. There is a minor CNS involvement (impairment of memory, impairment of visuospatial and constructional abilities). Periferal neuropathies include especially the facial nerves (Figure 1). We aim to study this disease using nanobody technology. This strategy is in principle applicable to many other neurological disorders.

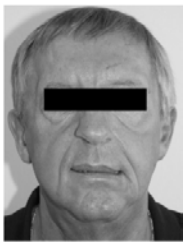

Figure 1. Bilateral upper and lower facial palsy in a FAF patient, caused by amyloid deposition in facial nerves. Reproduced from Luttmann et al., 2010.

2. The molecular basis of the disease

A point mutation (G654A/T) (de la Chapelle et al., 1992) in the **gelsolin** gene results in replacement of an aspartate residue (D187N/Y) that is crucial for calcium binding (Figure 2). As a result, **furin** proteolytically removes the N-terminal segment of gelsolin *en route* through the *trans* Golgi network, leaving a **68 kDa** C-terminal fragment (C68) (Chen *et al.*, 2001). Immediately upon secretion of C68, **MT1-MMP**, a metalloprotease, catalyzes formation of **8 and 5 kDa gelsolin peptides** that associate spontaneously into amyloid fibrils (Page *et al.*, 2005)(Figure 2) and accumulate in cranial nerves. **A mouse model** recapitulating the affliction (Page et al., 2009) is available in our lab.

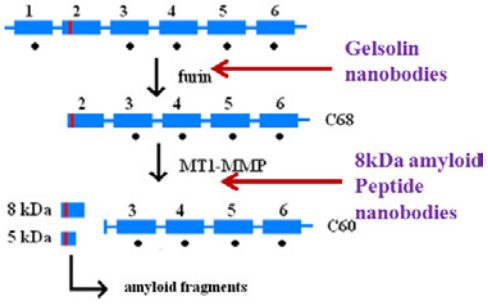

Figure 2. Mutant gelsolin domain 2 (red vertical line) is unable to bind calcium (black circle) and is cleaved twice during secretion, by furin and MT1-MMP, resulting in 8 kDa & 5 kDa peptides.

Nanobodies or VHHs are the smallest intact antigen-binding fragments from heavy chain antibodies present in serum of *Camelidae* species (Muyldermans *et al.*, 2009). Our work has shown that nanobodies act as *bona fide* antagonists of structural and catalytic proteins alike. We have raised nanobodies against a variety of cytoskeletal proteins (De Ganck et al., 2008; Delanote et al., 2010; De Clercq et al., 2012; Van Impe et al., 2008; Van den Abbeele et al., 2010. Nanobodies not only represent a useful research instrument but they also mimic the activity of drugs by interfering with protein function.

3. Results obtained during the previous year.

We raised 3 classes of gelsolin nanobodies (Figure 3): those that bind to the N-terminal half of gelsolin or the C-terminal half of gelsolin (Van den Abbeele et al., 2010), and another that interacts with the 8 kDa gelsolin peptide.

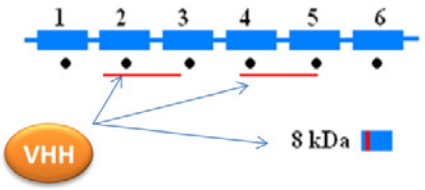

Figure 3. Schematic, showing where in gelsolin the 3 sets of nanobodies bind. The horizontal red lines indicate the epitope determined by pull down assays using GST fusions of gelsolin fragments and nanobodies, by approximation (Van den Abbeele et al., 2010).

3.1. Curtailing formation of amyloid peptides using a strategy involving indirectly blocking MT1-MMP activity (Van Overbeke et al., 2014).

MT1-MMP is a membrane-bound protease and Figure 2 (supra) shows that this protease is involved in formation of the 8 kDa gelsolin peptide that aggregates into fibrils.

We reasoned that selected gelsolin nanobodies could be endowed with the property of shielding the cleavage site, hence protecting further degradation of gelsolin. We set out to test this hypothesis *in vitro* as well as in vivo, making use of our gelsolin amyloidosis mouse model wherein the proteolytic cascade is recapitulated (Page et al., 2009).

What is further described in this section was presented this year in a paper, published in Molecular Therapy (Van Overbeke et al., 2014). The story was picked up by the *Knack* editorial office and featured as a story in the science section (a document is included in this report).

We developed nanobodies against the 8 kDa peptide and used these as molecular chaperones to mitigate gelsolin amyloid buildup. We identified gelsolin nanobodies that potently reduce C68 proteolysis by MT1-MMP *in vitro* (Left, Figure 4). We termed them FAF nanobodies 1-3.

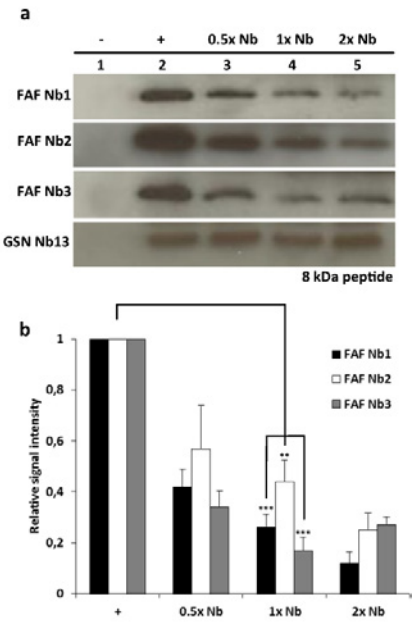

Figure 4 (a) FAF Nb1-3 were incubated with C68 (molar ratio nanobody:C68 indicated on top) prior to addition of MT1-MMP. The 8 kDa peptide was detected with anti-His HRP coupled antibody. Lane 1; negative control without MT1-MMP; lane 2: positive control with MT1-MMP but without addition of a nanobody. Increasing concentrations of nanobody (lanes 3-5, molar ratios indicated on top) progressively reduce 8 kDa peptide formation. Control GSN Nb13 has no effect on MT1-MMP cleavage of C68. **(b)** Quantification of data shown in **a**.

With a view of using these nanobodies in vivo, we needed to convert them into a format prolonging their lifetime. Indeed, nanobodies are rapidly cleared via the kidneys, rendering them inapt for medium or long term therapeutic purposes. We coupled FAF Nb1-3 to MSA21, an albumin binding nanobody. The effect of MSA21 linkage on the serum half-life was investigated by intraperitoneal injection of 100 µg recombinant nanobody into wild type C57BL/6 mice. Western blot analysis on blood samples taken at different time points revealed that the monospecific nanobody was nearly completely cleared from the circulation after four hours whereas the bispecific FAF Nb2-MSA21 remained stable in plasma up to one week post-injection, and was not degraded (Figure 5). Importantly, modifying the FAF nanobodies from a monospecific into a bispecific format did not affect their ability to reduce MT1-MMP catalyzed degradation of C68.

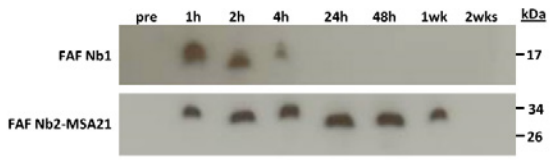

Figure 5 Wild type C57BL/6 mice were injected intraperitoneally with 100 µg V5-tagged FAF Nb1 or FAF Nb2-MSA21. Blood samples were taken at the indicated time points. Plasma was fractionated by SDS-PAGE followed by Western blotting. Nanobody was detected with polyclonal anti-V5 antibody.

To verify if the bulky FAF nanobody, coupled to MSA21, binds C68 *in vivo*, we performed immunohistochemistry on muscle tissue. A FAF heterozygous mouse (5.5 months of age) was injected with FAF Nb1 or FAF Nb2-MSA21 (both V5-tagged) and 1 hour later a musculus gastrocnemius tissue sample was obtained and stained for gelsolin (anti-8 kDa antiserum) or nanobody (anti-V5). The circumferential myofiber staining pattern observed for gelsolin is very similar to what has been reported earlier (Page et al., 2009) (Figure 6a,b). Importantly, the V5 nanobody staining pattern was identical to the gelsolin pattern for both the mono- and bispecific nanobody, (Figure 6c,d) indicating that both nanobody formats penetrate into muscle tissue and colocalize with gelsolin deposits or truncated gelsolin (Figure 6e,f). Thus, the nanobodies interact with their target after intraperitoneal injection.

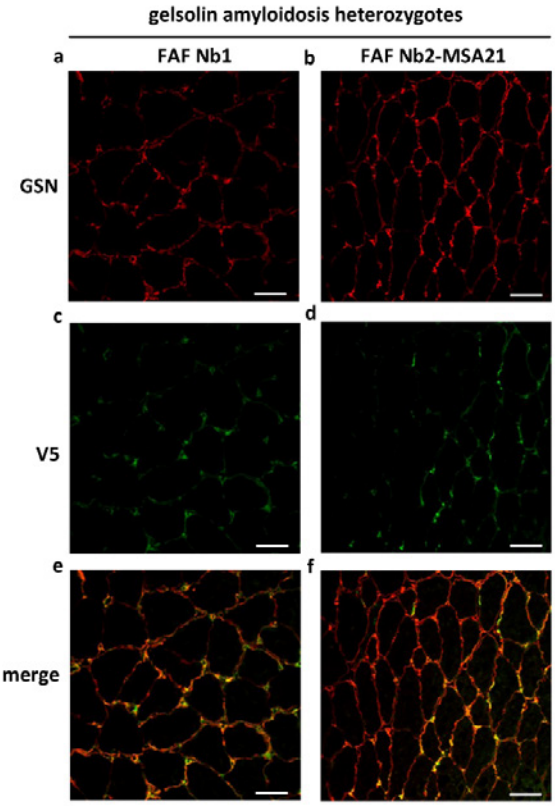

Figure 6: Intraperitoneally injected FAF Nb1 and FAF Nb2-MSA21 co-localize with gelsolin deposits in the endomysium of heterozygous gelsolin amyloidosis mice. Confocal microscopy images of heterozygous 5.5 month old gelsolin amyloidosis mice injected with 100 µg V5-tagged FAF Nb1 or FAF Nb2-MSA21. Musculus gastrocnemius tissue was dissected 1 hour post-injection and cryosections were stained for gelsolin and nanobody. **(a,b)** Gelsolin staining reveals a pattern that surrounds the myofibers. **(c,d)** V5 staining indicates presence of the nanobodies between the muscle fibers. **(e,f)** Merged images indicate co-localization between injected nanobody and gelsolin deposits in gelsolin amyloidosis mice. Scale bar = 50 µm).

To assess if these nanobodies trigger a therapeutic response in gelsolin amyloidosis mice, we set up an experiment in which heterozygous mice were injected weekly (IP) with 100 µg of recombinant nanobody. A control group of heterozygous mice was injected with 200 µL PBS at the same time intervals. To investigate their effectiveness, *in vitro* contractile function of 2 different hind leg muscles (extensor digitorum longus (EDL) and soleus) was evaluated by repeated electrically stimulated tetanic contractions of the intact incubated muscles. Typical features of contractile fatigue, such as the decrease in relaxation rate (Figure 7a) were attenuated in EDL of FAF Nb2-MSA21 injected mice, compared to PBS. Musculus gastrocnemius was dissected and staining showed to be more homogenous around the muscle fibers in PBS injected mice (Figure 7c, left panel). By contrast, the pattern was more focal and less intense in nanobody injected mice (Figure 7c, middle and right panels). Hence we can conclude that nanobodies reduce amyloid deposits around muscle tissue and improve its contractile properties.

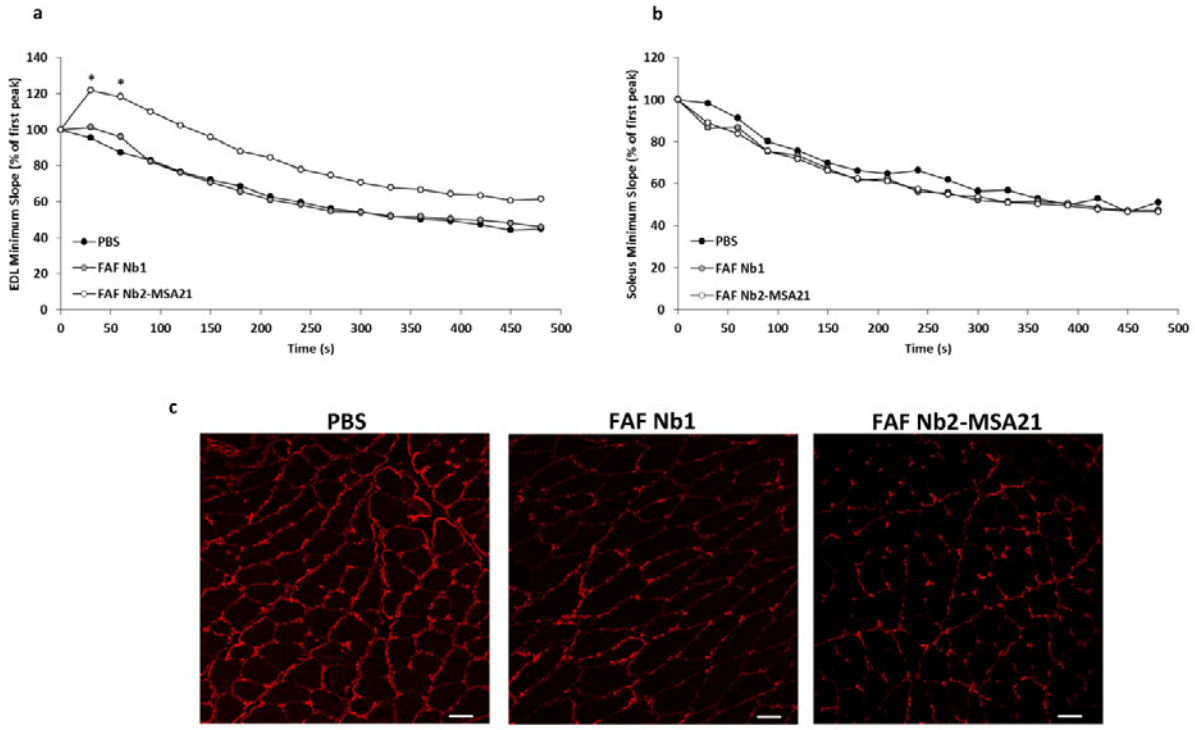

Figure 7. Nanobody injection in gelsolin amyloidosis mice significantly improves muscle contractile properties and reduces pathological gelsolin buildup in the endomysium. (**a**,**b**) Repeated *in vitro* muscle contractions in FAF Nb1 (grey circles) and FAF Nb2-MSA21 (white circles) injected mice compared to PBS controls (black circles). Injections with FAF Nb2-MSA21, but not with FAF Nb1, resulted in an attenuation of the slowing of the relaxation rate during fatigue in EDL (**a**) but not in soleus (**b**), * $p < 0.05$ FAF Nb2-MSA21 vs. PBS (two sided unpaired t-test). (**c**) Confocal microscopy images show sections of dissected musculus gastrocnemius, taken from gelsolin amyloidosis mice injected with PBS (left), FAF Nb1 (middle) or FAF Nb2-MSA21 (right).

3.2. Preventing gelsolin degradation by furin in transgenic mice (Van Overbeke et al., 2015)

Thanks to epitope mapping experiments in an earlier study (Van den Abbeele et al., 2010), we were also able to set up strategy where we aim to prevent the first cleavage step of gelsolin, by furin. The epitope of gelsolin Nb11 resides in gelsolin domain 2 (^{137}G – ^{247}L), encompassing the region where furin proteolyzes mutant plasma gelsolin (^{169}RVVR^{172}↓). We hypothesized that binding of this nanobody to mutant plasma gelsolin might interfere with furin proteolysis. GSN Nb13, interacting with gelsolin domains 4-5 (distant to the furin cleavage site), was chosen as a control. As a first step to verify our hypothesis we performed an *in vitro* furin cleavage assay. In the presence of GSN Nb11 (Figure 8, upper panels) we observed reduced proteolysis of full length plasma gelsolin as the molar ratio of Nb : gelsolin increased. GSN Nb13 (lower panels) had no influence on furin activity.

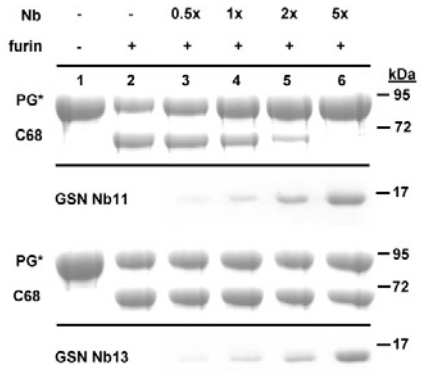

Figure 8. *In vitro* furin cleavage assay in which addition of furin to mutant plasma gelsolin (PG*) generates C68. Pre-incubation of GSN Nb11 (upper panel) with PG* reduced the amount of C68 generated by furin proteolysis in a concentration dependent manner. Negative and positive controls were included (lanes 1 and 2, respectively). Molar ratio nanobody:PG* is indicated by 0.5x – 5x in lanes 3-6. GSN Nb13 (lower panel) that binds gelsolin domains 4-5, irrelevant to furin proteolysis, had no reducing effect on C68 formation (lanes 3-6).

The gelsolin G2:Nb11 crystal structure provides insight into this mechanism

Together with the Robinson lab we performed crystallography on the G2 (^{159}V- ^{259}D):Nb11 complex (Figure 9, 2.6 Å resolution). This revealed that Nb11 does not directly block the furin cleavage site at Ala-173 but binds the short α–helix of the G2 domain. All of the Nb11 complementarity-determining regions (CDR) contribute hydrogen bonds to the interface with G2, while CDR1 and CDR3 also participate in hydrophobic interactions with G2, and CDR3 forms salt bridges with G2.

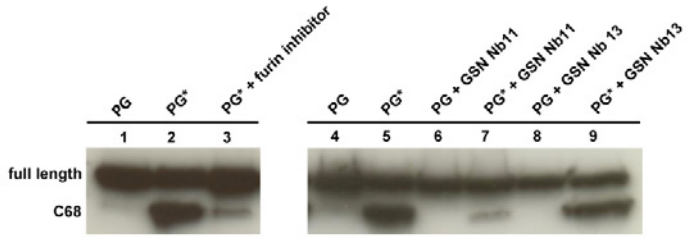

Figure 9. GSN Nb11 binds G2 at a distant site relative to the furin cleavage site (A173). Calcium is shown as a dark grey sphere; D187 is the gelsolin amyloidosis mutation site. Furin cleavage site (A173) and MT1-MMP cleavage sites (R225 and M243) are shown in ball-and-stick representations.

GSN Nb11 retains the ability to reduce furin proteolysis of PG* when directed to the trans-Golgi network of HEK293T cells

We further elaborated our hypothesis by testing the effect of GSN Nb11 in HEK293T cells. Since furin is active in the *trans*-Golgi network and therefore inaccessible to the nanobodies, we directed GSN nanobodies to the secretory compartment where they can bind PG* prior to encountering furin. When we analyzed the cell medium by western blot, we confirmed what had been observed (Figure 10, right panel). Transfection of GSN Nb11 (right panel, lane 7) drastically reduced C68 formation, comparable to the effect of furin inhibitor I (left panel, lane 3). GSN Nb13 did not influence C68 formation (right panel, lane 9). Wild type gelsolin was not observed to be proteolyzed (right panel, lanes 4, 6, 8).

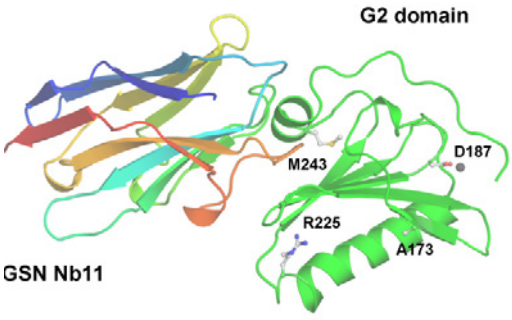

Figure 10. Western blot analysis of transfected HEK293T cell medium. Full length PG (left panel, lane 1), PG* and C68 (left panel, lane 2) are clearly detectable in the cell medium. 100 µM furin inhibitor I in the cell medium results in reduced C68 secretion (left panel, lane 3). Lanes 4-5 (right panel) show control signals for PG*/C68 secretion without GSN nanobody expression. Lanes 6 and 8 show wild type PG secretion without proteolysis when co-transfected with GSN Nb11/13. The effect of GSN Nb11 and GSN Nb13 on C68 formation is detectable in lanes 7 and 9, respectively. GSN Nb11 strongly reduces C68 formation whereas GSN Nb13 has no influence.

Development of a gelsolin nanobody secreting gelsolin amyloidosis mouse model

Transgenic gelsolin amyloidosis mice (Page et al, 2009) express human mutant plasma gelsolin and the furin cleavage product C68 is found in the plasma of these mice. We developed mice expressing ER-directed GSN Nb11/13 for subsequent cross breeding with the gelsolin amyloidosis mice (Figure 11). GSN amyloidosis/nanobody double positive mice will express both transgenic proteins and in this manner, GSN Nb11/13 will encounter PG* in the secretory compartment. ER-directed GSN Nb11/13 cDNA was cloned in the pROSA-DV2 vector, a vector targeting the ROSA26 locus (Figure 11**A**). After electroporation of the targeting vector in G4 ES cells, we checked for positive clones by Southern blotting (Figure 11**B**). Positive colonies were transfected with the cre-recombinase and mRNA was isolated from these cells to check for nanobody presence at the RNA level (Figure 11**C**). Nanobody positive ES cells were aggregated with Swiss inner cell mass (ICM) cells and the resulting blastocysts were transferred to the uteri of pseudopregnant Swiss fosters which resulted in a chimeric offspring (Figure 11**D**). This offspring were backcrossed with wild type C57BL/6 to check for germline transmission (Figure 11**E**). Pups with nanobody cDNA in the germline cells were crossed with *Cre* deleter mice to remove the floxed STOP-cassette and activate transcription of the nanobody cDNA (Figure 11**F**). Offspring were genotyped for nanobody and Cre and Nb/Cre double positive pups were checked for nanobody presence in the plasma by co-immunoprecipitation and Western blot analysis (Figure 11**G**). In a final step, these nanobody mice were crossed with gelsolin amyloidosis homozygotes to create GSN amyloidosis/nanobody double positive mice. As a final verification, the offspring were genotyped for gelsolin amyloidosis and checked for nanobody expression in the plasma (Figure 11**H**).

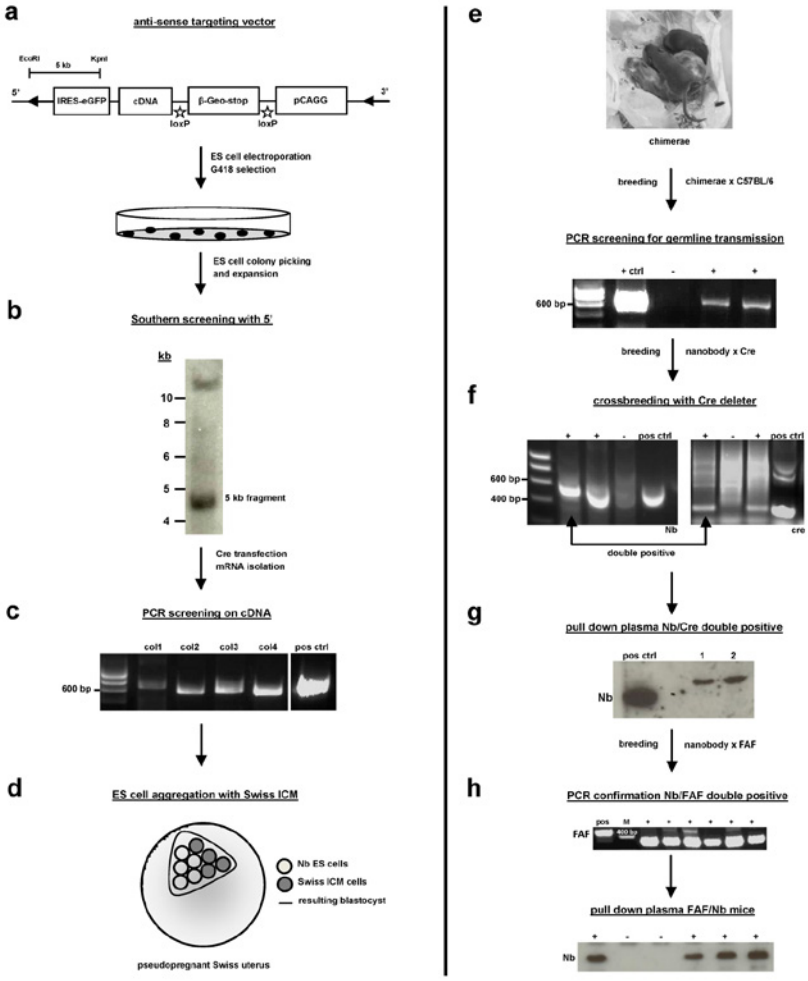

Figure 11 Development of transgenic, GSN Nb11/13 expressing gelsolin amyloidosis mice. (a) A pROSA26-DV2 targeting vector containing the ER-directed GSN Nb11/13 was developed to insert the GSN nanobodies in the murine genome at the ROSA26 locus. **(b)** Successful integration in the ES cell genome was confirmed by Southern screening. **(c)** cDNA was used a template for a PCR analysis using nanobody specific primers. **(d)** Positive ES cell clones were aggregated with Swiss ICM cells to form a blastocyst overnight. The resulting blastocyst was transferred to the uterus of a pseudo pregnant Swiss female. **(e)** Chimerae were bred with wild type C57BL/6 mice to check for germline transmission **(f)** To remove the STOP-cassette, the mice were crossed with Cre deleter mice. **(g)** Offspring from this breeding were checked for GSN Nb11/13 expression at the protein level by means of immunoprecipation from plasma and Western blot analysis. **(h)** Mice that were positive at this stage were finally crossed with homozygous FAF mice

Counteracting furin proteolysis by GSN Nb11 results in improved muscle contractile properties
To test if the altered gelsolin staining patterns had an influence on muscle functionality, we examined muscle contractile properties of the transgenic mice. Typical features of a fatiguing protocol are a decrease in force development and reductions of contraction and relaxation speed. The decrease in contraction speed was strongly attenuated, across the entire 8-minutes fatigue protocol, in EDL, but not in soleus of the GSN Nb11 mice compared to littermate controls (Fig. 12a,b). Hence, as in the previous approach, we can improve physiological muscle performance of these lab animals using nanobody technology.

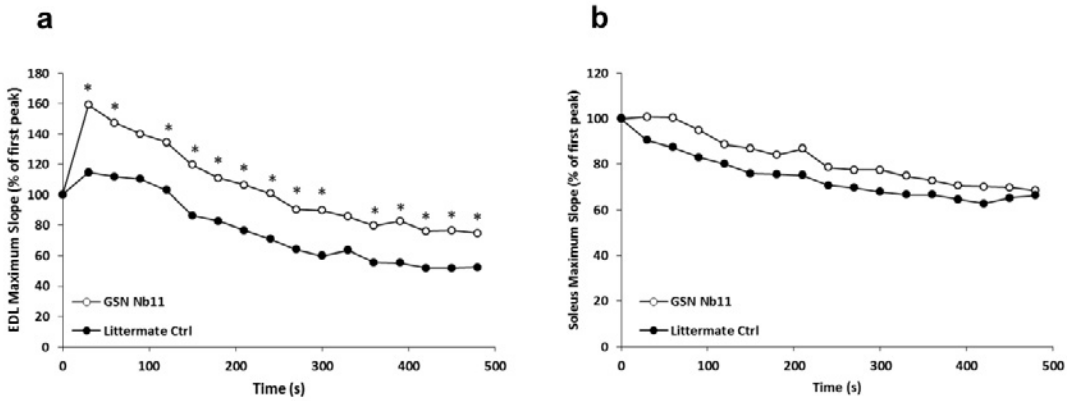

Figure 12. **Muscle contractile properties of gelsolin Nb11 expressing heterozygotes and their littermate controls.** Repeated *in vitro* muscle contractions in GSN Nb11 gelsolin amyloidosis mice (white dots) compared to littermate controls (black dots). (a,b) GSN Nb11 expression resulted in an attenuation of the slowing of contraction speed during fatigue in EDL (A), but not in soleus (B), * $p < 0.05$ GSN Nb11 (n = 5) vs. littermate controls (n = 5).

The paper describing these findings was submitted to *Human Molecular Genetics* (Van Overbeke et al., 2015) We have received a favorable review and the revised version will be submitted in the first half of January. We expect that this second paper will then be accepted.

3.3. Visualization of amyloid fibrils in diseased animals.

A third important goal in this project was to study disease progression in the same animal over time in a non-invasive manner. We are very fortunate because also here we are making very good progress, thanks to the collaboration with the lab of **prof. T. Lahoutte** (*In Vivo Cellular and Molecular Imaging Laboratory, Free University Brussels*). Following ^{99m}Tc labeling of a gelsolin anti-8kDa peptide nanobody, in combination with *single-photon emission computed tomography/computed tomography* (SPECT/CT) imaging, we performed biodistribution and imaging studies. We traced gelsolin peptides following peritoneal injection of the nanobody tracer (Figure 13). Drs. Verhelle from our lab, and who is executing these experiments, has visited the Lahoutte lab on several occasions and has also attended a three day training course in Brussels to become more acquainted with SPECT/CT imaging.

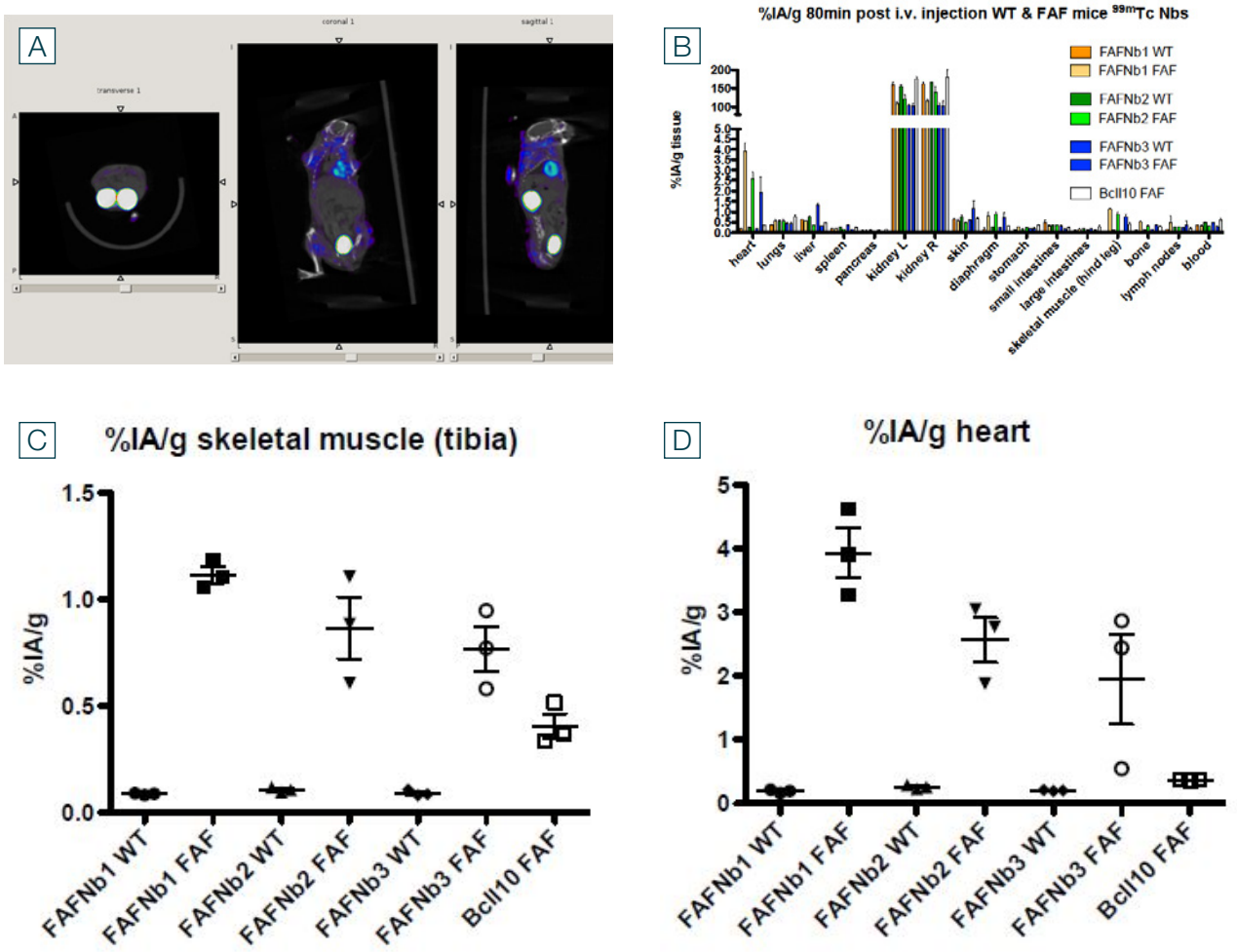

Figure 13. A) Representative SPECT/CT recording of a 9months old FAF C57BL/6 mouse using ^{99m}Tc-FAF Nb1. The coronal section (middle) clear shows skeletal muscle, heart and bladder. The sagittal section (right) also highlights the kidneys. B) Biodistribution of the FAF anti peptide Nbs in 9 months old FAF C57BL/6 animals compared to the biodistribution in wild type C57BL/6. FAF Nbs do not accumulate non-specifically. C) Specific uptake in the muscle in WT vs. FAF animals at 9 months of age. D) Specific uptake in the heart in WT vs. FAF animals at 9 months of age.

This work is not completed yet and a longitudinal study is ongoing. We expect to complete this part of the project in the course of this year. But it shows for the first time the possibility to use nanobodies for imaging of amyloid fibrils.

3.4. Can we protect gelsolin against furin and MT1-MMP simultaneously?

The possibility to link nanobodies in tandem array format allows us to investigate if we can prevent furin **and** MT1-MMP mediated hydrolysis using a single construct consisting of two different nanobodies.

In our previous study we reared transgenic mice but in the mean time our experience has shown that it is difficult to guarantee a high expression level of the nanobody when it is inserted into the ROSA26 locus of embryonic stem cells. We therefore plan to use AAV-vectors in the future (adeno associated viral particles).

Such vectors are an efficient tool to obtain a very high level of transduction in vivo. The transgene remains episomal for several months. Furthermore, there is quite some diversity in selection of serotypes which enables us to target different tissues. Serotype 8 for instance has a very high transduction efficiency in skeletal muscle.

We have already generated a tandem construct consisting of gelsolin nanobody 11 followed by FAF nanobody 1, separated by a linker sequence. We are currently testing to what extent this construct protects FAF mutant gelsolin against MT1-MMP *and* furin. We need to optimize the assay conditions because both proteases are active in entirely different buffer conditions. For AAV production we have initiated a **collaboration with prof. Marinee Chuah and prof. Thierry Vandendriessche (**Department of Gene Therapy & Regenerative Medicine, Free University Brussels). They are experts in therapeutic approaches involving viral technologies.

4. Other noteworthy events

- Both students working on this project, Drs. Wouter Van Overbeke and Drs. Adriaan Verhelle, *received a travel stipend* from the *United States Amyloidosis formation*, valued at 2500 $ each, to participate at the **The XIVth International Symposium on Amyloidosis** in Indianapolis, Indiana from 27 April to 1 May, 2014.
- At this meeting, Drs. Van Overbeke was *awarded* the *Junior Investigation Award* by the International Society of Amyloidosis.
- On Friday the 16th of January 2015, Drs. Van Overbeke will defend his PhD work in public, entitled: **Keeping mutant plasma gelsolin safe from harm: gelsolin nanobodies act as a chaperone against pathological proteolysis.**

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6. Publications directly supported by GSKE:

- Van Overbeke W, Verhelle A, Everaert I, Zwaenepoel O, Vandekerckhove J, Cuvelier C, et al. Chaperone nanobodies protect gelsolin against MT1-MMP degradation and alleviate amyloid burden in the gelsolin amyloidosis mouse model. *Mol Ther.* 2014;22:1768-78.
- Van Overbeke W, Wongsantichon J, Everaert I, Verhelle A, Zwaenepoel O, De Ganck A, Hochepied T, Haigh J, Cuvelier C, Derave W, Robinson R, Jan Gettemans. An ER-directed nanobody targets the first step in amyloid formation in a hereditary gelsolin amyloidosis mouse model by protecting mutant plasma gelsolin from furin proteolysis. Revision completed. 2015. MS to be resubmitted shortly. *Human Molecular Genetics.*

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Progress report of the research group of

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Decoding spontaneous mnemonic brain activity during post-training wakefulness and sleep using high-density EEG and electro-corticography

Introduction

During this year, our research followed 4 related streams:
- The regulation of sleep/wakefulness rhythms
- The non-visual influence of light upon cognitive brain responses
- The genetic influences on executive functions.

These 3 research streams are detailed in the following sections.

1. 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. To address this issue, we conducted an fMRI study during which 35 normal volunteers were serially scanned during a 42-hour sleep deprivation under constant routine conditions.
The data are being analyzed and preliminary results show that brain response to a simple reaction time task is modulated by both circadian rhythm and sleep homeostasis.

2. Non visual responses to light influence brain cognitive responses

We conducted an fMRI study which is based on physical properties of melanopsin, the photopigment that conveys the non-image forming visual responses (figure 1).

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.

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This article was “Research Highlight” in Nature (vol 507:276; March 20th 2014)

3. The genetic influences on executive functions

Genetic variability related to the catechol-O-methyltransferase (COMT) gene (Val(158)Met polymorphism) has received increasing attention as a possible modulator of cognitive control functions. METHODS: In an event-related functional magnetic resonance imaging (fMRI) study, a modified version of the Stroop task was administered to three groups of 15 young adults according to their COMT Val(158)Met genotype [Val/Val (VV), Val/Met (VM) and Met/Met (MM)]. Based on the theory of dual mechanisms of control (Braver et al., 2007), the Stroop task has been built to induce proactive or reactive control processes according to the task context. RESULTS: Behavioral results did not show any significant group differences for reaction times but Val allele carriers individuals are less accurate in the processing of incongruent

items. fMRI results revealed that proactive control is specifically associated with increased activity in the anterior cingulate cortex (ACC) in carriers of the Met allele, while increased activity is observed in the middle frontal gyrus (MFG) in carriers of the Val allele. CONCLUSION: These observations, in keeping with a higher cortical dopamine level in MM individuals, support the hypothesis of a COMT Val(158)Met genotype modulation of the brain regions underlying proactive control, especially in frontal areas as suggested by Braver et al.

– **Jaspar, M., Genon, S., Muto, V., Meyer, C., Manard, M., Dideberg, V., Bours, V., Salmon, E., Maquet, P. & Collette, F. 2014. Modulating effect of COMT genotype on the brain regions underlying proactive control process during inhibition. *Cortex,* 50, 148-61.**

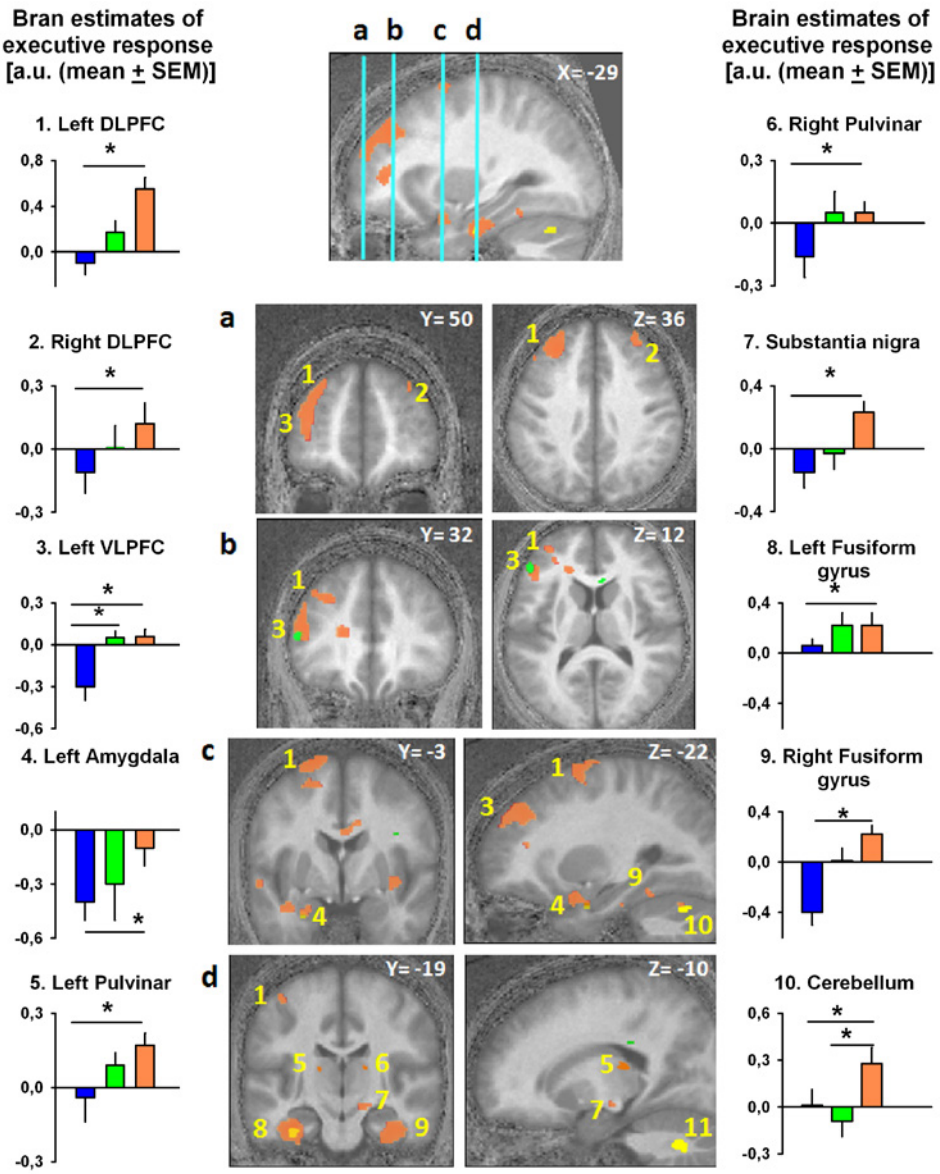

Figure 1. Impact of test light on executive brain responses depends on prior light.
Orange blobs: increased test light impact following prior orange relative to prior blue light exposure.
Green blobs: increased test light impact subsequent to prior green relative to prior blue light.
Yellow blobs: increased test light impact following prior orange relative to prior green light.
Graphs: Brain activity estimates of test light impact on executive responses (3-back – 0-back; arbitrary units [a.u.] mean ± SEM) following exposure to blue, green and orange light. 1-2.Left and right dorsolateral prefrontal cortex (DLPFC); 3. Left ventrolateral prefrontal cortex (VLPFC); 4. Left amygdala (AMY); 5-6. left and right pulvinar (PUL); 7. Substantia nigra (SN); 8-9. Left and right fusiform gyrus (FUS); 10. Cerebellum (CER).
** p <0.05 corrected for multiple comparisons.*

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

Progress report of the research group of

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System x_c^- as a potential target for novel neuroprotective strategies: focus on Parkinson's disease and its psychiatric comorbidities

1. Introduction on Parkinson's disease and its psychiatric comorbidities

In Parkinson's disease (PD), nigrostriatal degeneration causes a loss of dopamine in the striatum. As a result, the motor loop of the basal ganglia is dysregulated, resulting in hyperactivity of the subthalamic nucleus (STN) and subsequent overactivaton of the GABA-ergic output structures (i.e. substantia nigra pars reticulata (SNr), globus pallidus pars interna (GPi)) which in turn inhibits the thalamus. This results in motor impairment (bradykinesia, tremor and rigidity) due to less excitatory input from the thalamus to the motor cortex. The hyperactive glutamatergic STN neurons also project to the dopamine containing neurons located in the substantia nigra pars compacta (SNc) and may cause more neurodegeneration due to toxic glutamate levels (excitotoxicity). As such, SNc neuronal loss and STN overactivation sustain each other and may cause progression of the disease[1,2]. In this respect, the use of NMDA (ionotropic glutamate receptor) antagonists is clearly beneficial as neuroprotective effects can be observed. However, their use is hampered by neurological side effects that are the consequence of impairment of fast excitatory NMDA-mediated synaptic transmission[3,4]. Moreover, as recently described, the stimulation of extrasynaptic NDMA receptors triggers cell destruction pathways whereas the stimulation of synaptic NMDA receptors is involved in neuroprotection[5,6]. Antagonists of the postsynaptic group I metabotropic glutamate receptor (mGluR) mGluR5, have also been shown to be neuroprotective in the absence of overt side effects, as they negatively modulate NMDA responses without completely blocking synaptic transmission[7-9].

Besides the classical motor symptoms, 65% of PD patients suffer from neuropsychiatric symptoms such as depression and anxiety. The underlying mechanisms of depression and anxiety in PD are still unclear and might be attributed to a combination of medical, neurochemical and psychosocial factors. Interestingly, both depression and anxiety may precede PD onset, indicating they are not merely the result of the difficulties related to PD[10].

Despite therapeutic advances over the last decades, PD can still only be treated symptomatically. Moreover, medication that could treat the neuropsychiatric comorbidities, such as antidepressants or anxiolytic agents, have not been proven to be effective in PD and the risk of deterioration of PD as well as interactions with the PD medication are a major concern[10]. In conclusion, new insights into the molecular mechanisms leading to PD and its comorbidities are crucial as they might provide new targets for disease-modifying interventions.

2. Project background information

System x_c^- or the Na^+-independent cystine/glutamate antiporter, consisting of xCT as a specific subunit and 4F2hc, is located on glial cells and imports one cystine molecule in exchange for a glutamate molecule in an obligatory 1:1 exchange rate[11] (fig. 1). Increased activity can as such contribute to excitotoxic damage. In the context of our research focus, i.e. the role of glutamate transporters in neurological disorders[12-16], we were the first to propose a possible involvement of system x_c^- in the pathogenesis of PD. This hypothesis is based on the observation that xCT expression levels were increased in the ipsilateral striatum of the unilateral 6-hydroxydopamine (6-OHDA) hemi-Parkinson rat model[17]. In order to understand the functional meaning of this increased xCT expression levels, we used mice with a genetic

deletion of xCT ($xCT^{-/-}$)[18] and tested their vulnerability for 6-OHDA-induced neurodegeneration. Although no effect was seen on striatal dopamine loss, the dopaminergic neurons in the SNc of these mice were significantly protected. Apart from the strong decrease in striatal extracellular glutamate levels, we could not observe neurochemical or anatomical changes in the $xCT^{-/-}$ brain under physiological conditions and therefore we concluded that the protective effects of the loss of system x_c^- should probably be linked to the decreased extracellular glutamate levels[19].

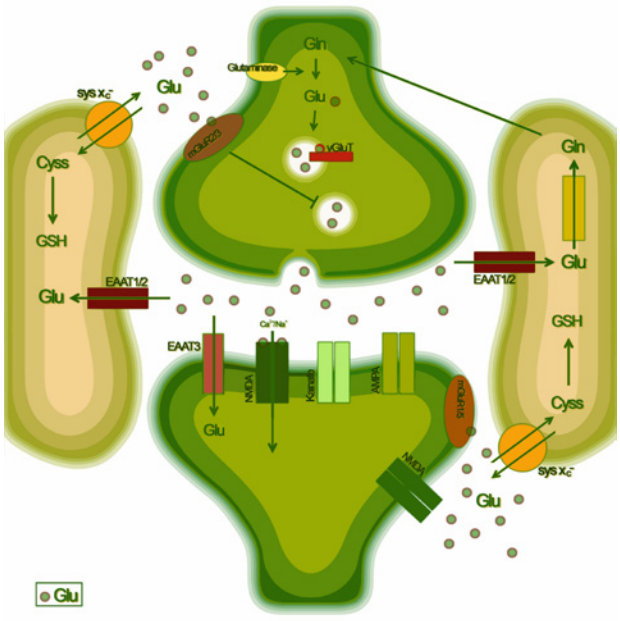

Figure 1: schematic representation of a glutamatergic synaps including glutamate reuptake transporters (EAAT), glutamate receptors (NMDA, AMPA, mGluR) and system x_c^- (sys x_c^-).

The **rationale for studying system x_c^- as a target for treating PD and its comorbidities** is based on the dual role this glial antiporter can have in neurological disorders that are characterized by increased levels of oxidative stress as well as excitotoxicity. Cystine is intracellularly reduced to cysteine, which is the rate limiting building block in the synthesis of glutathione (GSH), a major brain antioxidant. Consequently, the expression of system x_c^- can be induced by pathways that are activated by oxidative stress or other stress stimuli[20]. Indeed, we observed increased xCT expression in the ipsilateral striatum of the 6-OHDA hemi-Parkinson rat model[17]. However, each time a cystine molecule is imported, glutamate is released into the extracellular space and we demonstrated that under physiological conditions, in certain brain regions 60-70% of extracellular glutamate levels, as measured using *in vivo* microdialysis, originate from system x_c^- [16-19]. This **glutamate**, that is **released extrasynaptically**, can activate extrasynaptic NMDA receptors and mGluRs[21] and, as such, induce neurodegeneration when present in excess. **Our findings that mice lacking system x_c^- are protected against 6-OHDA-induced neurodegeneration[19] suggest 1/ that mice can perfectly deal with (oxidative) stress situations in the absence of system x_c^- and 2/ that avoiding the upregulation of system x_c^- in response to this cellular stress is protective, probably due to the fact that excessive glutamate release is prevented.** It thus seems that in certain pathological conditions, the brain induces excitotoxic damage in an attempt to protect itself against oxidative stress.

The goal of this project is to strengthen our hypothesis that inhibition of system x_c^- is a novel, neuroprotective strategy for the treatment of PD and its comorbidities. In order to do so, we proposed a validation plan consisting of four specific aims: **1/** investigate whether xCT expression levels are affected in PD-related structures of human PD patients, **2/** investigate the susceptibility of mice with a genetic deletion of xCT, and thus lacking functional system x_c^-, for lactacystin (LAC, inhibitor of the proteasome injected into the substantia nigra (SN)) and MPTP (1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine)-induced parkinsonism. Vice versa, we will investigate the effect of LAC- and MPTP-induced parkinsonism on the expression of xCT. **3/** We will unveil whether downregulation or inhibition of system x_c^- in the STN at a certain time in life and at a certain stage of the disease, instead of a genetic deletion, can still protect dopamine neurons from the SNc against toxin-induced degeneration. **4/** Ultimately, we will investigate the link between system x_c^- and anxiety/depression in healthy as well as parkinsonian mice.

3. Results obtained in 2014:

3.1. Specific aim 1: Investigate xCT mRNA (real-time PCR) and protein (semi-quantitative Western blotting) levels in tissue samples of PD patients and healthy controls.

Up till now, we only collected some preliminary data on human tissue samples (fig. 2). With a limited number of samples, we could observe a trend towards increased xCT protein expression in cortex of PD patients compared to healthy controls and a decreased xCT expression in the SN of PD patients. In the near future, additional samples will be obtained from the Netherlands brain bank.

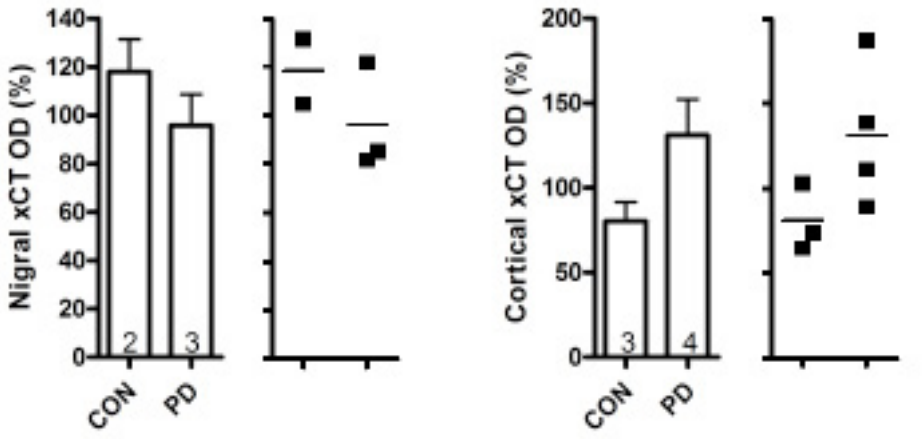

Figure 2: **Expression levels of xCT** in post-mortem nigral and cortical tissue from **PD patients** compared to healthy controls. Immunoblot analyses revealed a trend towards decreased expression in the SN and increased expression in the cortex. Data are presented as mean ± SEM. CON control subjects.

3.2. For specific aim 2 we investigated whether our observations in the 6-OHDA model can be generalized to other PD models, by studying xCT expression in the LAC/SN model and the chronic, progressive MPTP model. At the same time, we compared the behavioral and neurochemical outcome of $xCT^{-/-}$ and $xCT^{+/+}$ mice in both models.

Since we are the first to use a mouse model for PD in which LAC is injected into the SN, we further characterized this model before investigating the involvement of system x_c^- in LAC-induced parkinsonism. A very thorough behavioral and neurochemical analysis was performed on three month old C57Bl/6 mice, one and three weeks after LAC injection.

In the manuscript that is currently in revision (Bentea et al., Frontiers in Behavioral Neuroscience) we report that unilateral administration of LAC to the SN of mice leads to acute and non-progressive dopaminergic neurodegeneration (fig. 3), in the presence of increased levels of Ser129-phosphorylated α-synuclein (fig. 4). These pathological changes induced the development of motor asymmetry and impairment, as assessed in various motor behavior paradigms (fig. 5). Furthermore, we detected signs of non-motor symptoms resembling early-stage Parkinson's disease, including somatosensory disturbances, akathisia (restlessness), perseverative behaviour (fig. 5), and anxiety-like behaviour (fig. 6).

We conclude that the intranigral LAC mouse model can be a relevant model to study the involvement of proteasomal dysfunction and of authentically phosphorylated α-synuclein at Ser129 in the pathogenesis of sporadic Parkinson's disease.

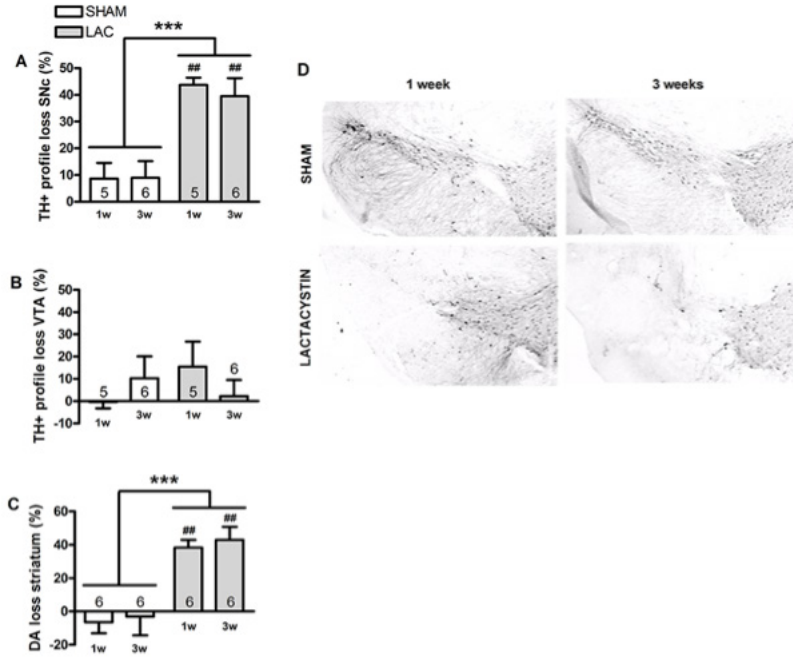

Figure 3: Significant **degeneration of the nigrostriatal DA-ergic pathway** in mice receiving 3µg LAC. TH immunohistochemistry revealed a significant loss of TH+ profiles in the SNc (A, D), but not the VTA (B), after LAC lesion compared to sham mice receiving vehicle. Loss of nigral TH+ profiles translated to reduced striatal DA content of the lesioned mice, compared to sham surgery, at both 1 week and 3 weeks post lesion (C). Data are presented as percentage TH+ profiles or DA content compared to the intact side (mean ± SEM), and analyzed using 2-way ANOVA, followed by Bonferroni *post-hoc* tests. ***p<0.001 (2-way ANOVA), ##p<0.01 (Bonferroni *post*-hoc vs. sham) (n=5-6 per group) (scale bar 50µm). DA dopamine, LAC lactacystin, TH tyrosine hydroxylase, SNc substantia nigra pars compacta, VTA ventral tegmental area.

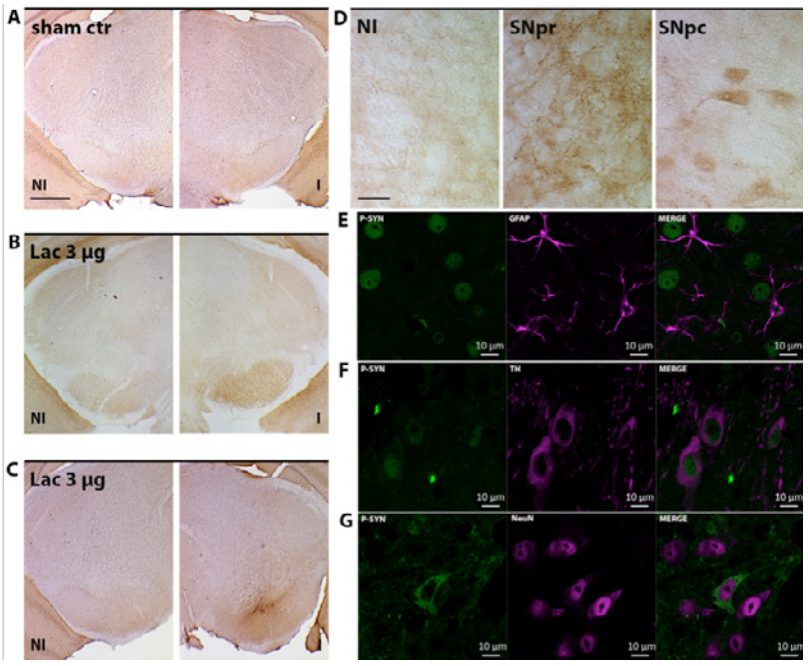

Figure 4: Accumulation of S129-P α-synuclein in mice receiving 3µg LAC. Immunohistochemical analyses revealed an increase in S129-P α-synuclein immuno-reactivity in the LAC injected SNc (B, C), that also affected the pars reticulata in a subset of mice (B). Sham-injected mice did not show visible expression of S129-P α-synuclein (A). High magnification photomicrographs of (B) demonstrate the presence of S129-P α-synuclein immunoreactive cells in the SNc, and fibers in the pars reticulata (D). Furthermore, fluorescent micrographs demonstrate accumulation of S129-P α-synuclein in nigral TH+ (F) and NeuN+ (G) neurons, but not GFAP+ astrocytes (E) (scale bar 200µm in A-C, 20µm in D, and 10µm in E-G). S129-P Ser129-phosphorylated, LAC lactacystin, SNc substantia nigra pars compacta, TH tyrosine hydroxylase, NeuN neuronal nuclei, GFAP glial fibrillary acidic protein.

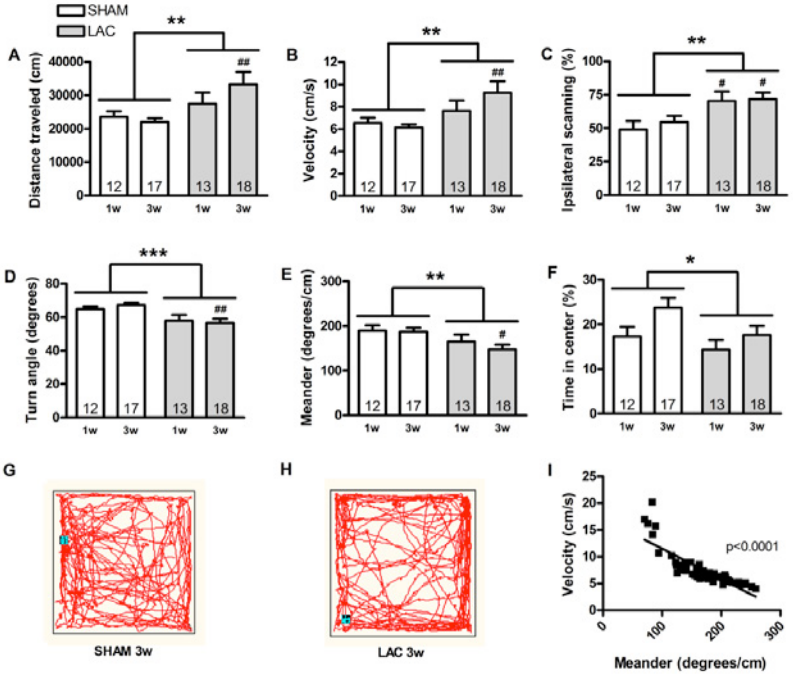

Figure 5: Spontaneous behavior of mice treated with 3µg LAC as observed in a 60min open-field test. Distance traveled (A), and velocity (B) were globally increased in LAC lesioned mice, compared to sham mice receiving vehicle. Furthermore, mice treated with LAC had significantly increased ipsilateral scanning behavior (C). LAC lesion also induced global decreases in turn angle (D), as well as in meander (E). This perseverative behavior is reflected in representative 10min trajectories of a sham treated (G), and a LAC treated (H) mouse at 3 weeks post-surgery. Linear regression analysis performed with data from all experimental groups revealed a close inverse correlation between meander (**perseverative behavior**) and velocity (**hyperkinesia**) (I). LAC lesion induced a global reduction in time spent in the center of the arena, compared to sham mice (F). Data are presented as mean ± SEM, and analyzed using 2-way ANOVA, followed by Bonferroni *post-hoc* tests. *p<0.05, **p<0.01, ***p<0.001 (2-way ANOVA), #p<0.05, ##p<0.01 (Bonferroni *post*-hoc vs. sham) (n=12-18 per group). LAC lactacystin

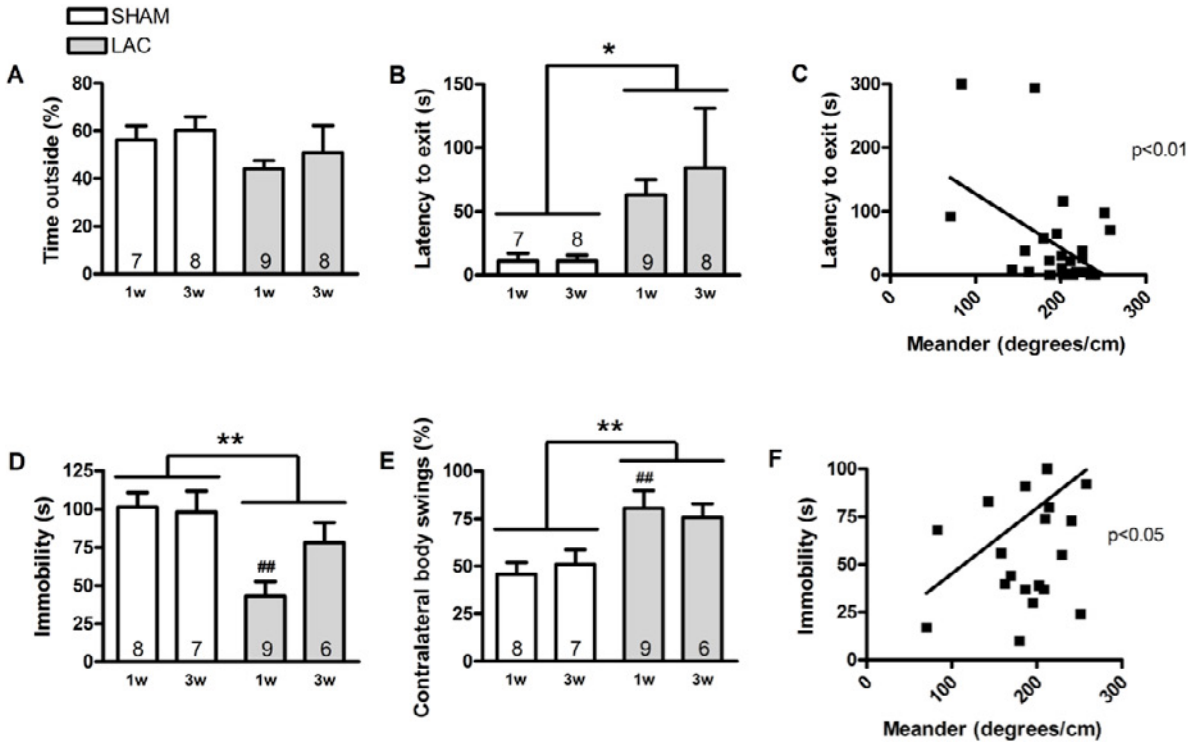

Figure 6: **Anxiety-like and depressive-like behavior** in mice receiving 3µg LAC. Although the time spent outside the shelter in the light/dark paradigm was not significantly affected (A), LAC lesion led to a global increase in the latency to exit the shelter compared to sham mice receiving vehicle (B). These changes occurred in the absence of significant changes in motor function, as the velocity outside the shelter was not affected by LAC lesion (data not shown). The tail suspension test revealed that LAC treated mice had decreased periods of immobility, compared to sham mice (D). Linear regression analysis performed with data from all experimental groups revealed that the "anxiogenic" effect (latency to exit the shelter) and the "anti-depressive"-like effect (immobility) correlated with perseverative behavior (meander) (C, F). Mice treated with LAC also demonstrated significantly more ipsilateral body swings compared to sham mice during their escape-oriented behavior (E). Data are presented as mean ± SEM, and analyzed using 2-way ANOVA, followed by Bonferroni *post-hoc* tests. *p<0.05, **p<0.01 (2-way ANOVA), ##p<0.01 (Bonferroni *post*-hoc vs. sham) (n=7-9 per group in A-C, n=6-9 per group in D-E). LAC lactacystin

We next investigated the involvement of system x_c^- in LAC-induced parkinsonism. xCT expression levels were measured in striatum of LAC- and sham-injected mice using semi-quantitative Western blotting (fig. 7). Preliminary data on a limited number of mice show a strong trend towards increased xCT expression levels in parkinsonian striatum. However, groups need to be enlarged and additional brain regions will be investigated in the coming months.

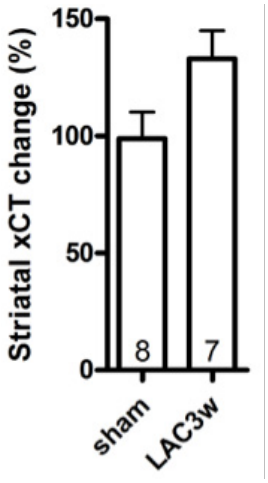

Figure 7: Expression levels of xCT in striatal tissue from LAC treated mice. Immunoblot analyses revealed a trend towards increased expression of xCT in the ipsilateral striatum of LAC-treated mice compared to sham-treated mice, at 3 weeks post lesion. Data are presented as mean ± SEM. LAC lactacystin.

Finally, the behavioral and neurochemical outcome of $xCT^{-/-}$ mice after LAC injection was compared to $xCT^{+/+}$ mice. In adult mice (three months old), we could not observe any differences in susceptibility for LAC-induced nigrostriatal degeneration (fig. 8) or behavioral impairment (fig. 9).

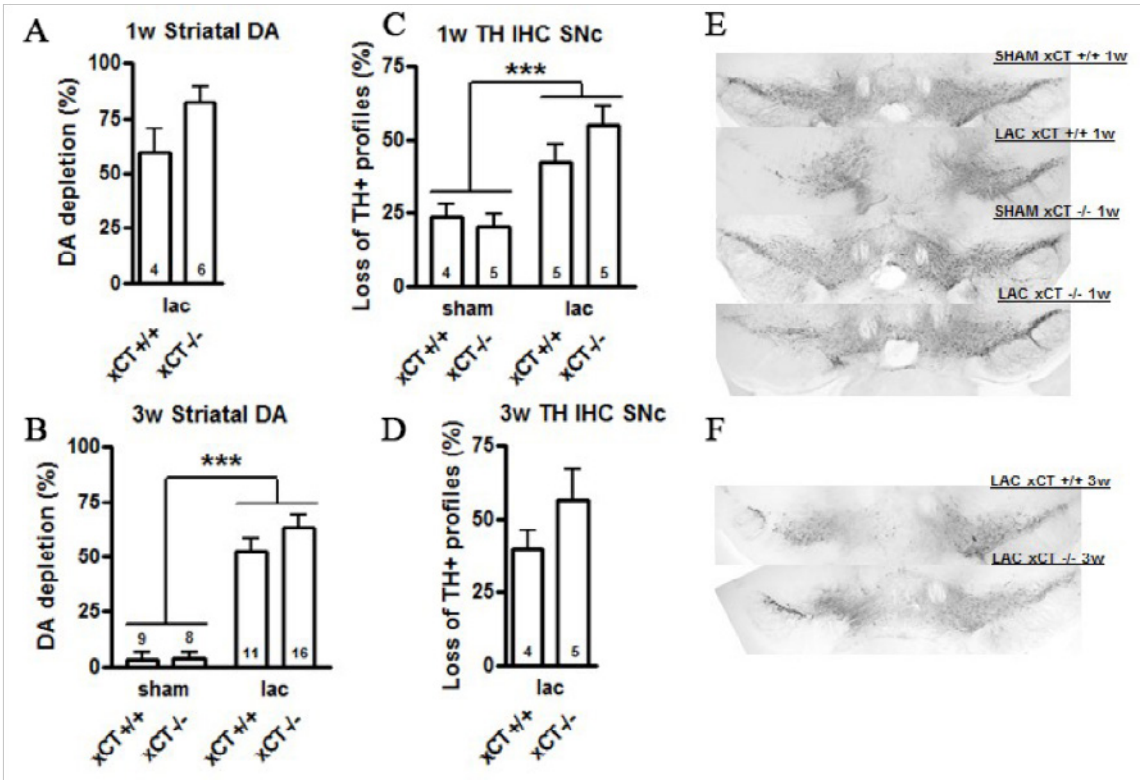

Figure 8: Adult $xCT^{-/-}$ mice demonstrate **similar nigrostriatal damage after LAC** compared to $xCT^{+/+}$ littermates. TH immunohistochemistry revealed a significant loss of TH+ profiles in the SNc after LAC lesion, that was statistically similar between $xCT^{-/-}$ and $xCT^{+/+}$ mice, at both 1w (C) and 3w (D) post-surgery (panels E and F shown representative TH photomicrographs). Loss of nigral TH+ profiles reduced striatal DA content to a similar degree in both genotypes, at either 1w (A) or 3w (B) post-surgery. Data are presented as percentage TH+ profiles or DA content compared to the intact side (mean ± SEM), and analyzed using 2-way ANOVA, followed by Bonferroni *post-hoc* tests. ***p<0.001 (2-way ANOVA). DA dopamine, LAC lactacystin, TH tyrosine hydroxylase, IHC immunohistochemistry SNc substantia nigra pars compacta.

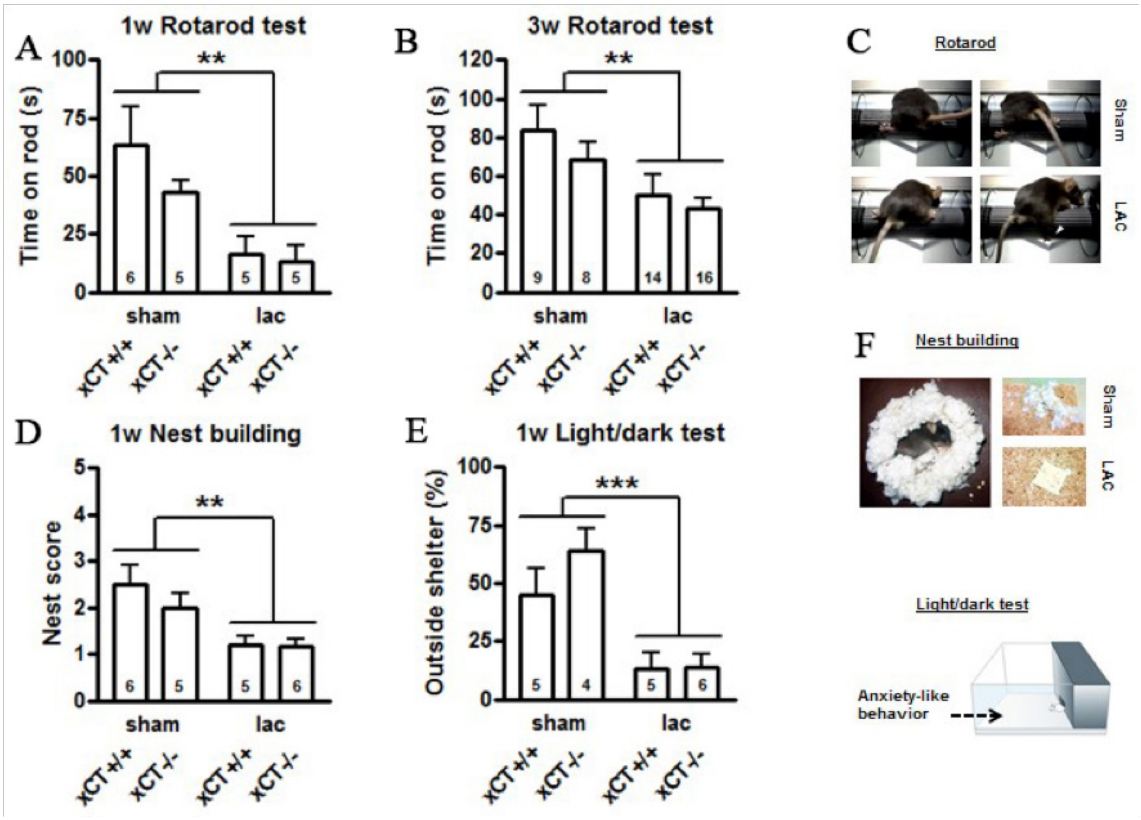

Figure 9: Adult xCT$^{-/-}$ mice show **similar motor and non-motor impairment after LAC** compared to xCT$^{+/+}$ littermates. LAC-induced nigrostriatal damage led to a loss of motor coordination and balance, as assessed in the rotarod test (C), that was equally observed in xCT$^{-/-}$ and xCT$^{+/+}$ mice, at 1w (A) or 3w (B) post-surgery. Furthermore, the quality to build a nest in the nest building test (as index of fine motor skills) (F) was similarly decreased after LAC for both genotypes, 1w after lesion (D). Finally, LAC lesion increased anxiety-like behavior at 1w post-lesion to a similar degree in xCT$^{-/-}$ and xCT$^{+/+}$ mice (E), as the time spent outside the shelter in the light dark test was not statistically different between the two genotypes. Data are presented as mean ± SEM, and analyzed using 2-way ANOVA, followed by Bonferroni *post-hoc* tests. ***p<0.001, **p<0.01 (2-way ANOVA). LAC lactacystin.

On the other hand, in aged mice (i.e. 19-23 month old mice) we could clearly observe neuroprotection as the result of the loss of system x_c^- (fig. 10).

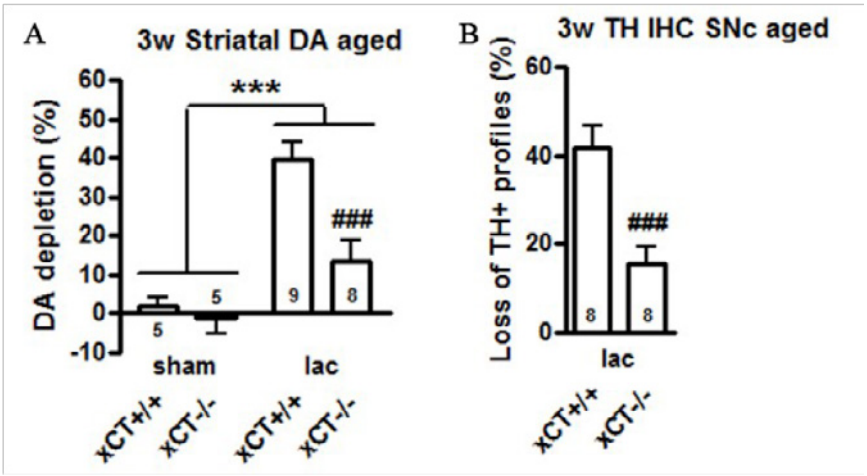

Figure 10: **Aged xCT$^{-/-}$ mice** are **protected from LAC-induced neurodegeneration** compared to xCT$^{+/+}$ littermates. TH immunohistochemistry revealed a significant loss of TH+ profiles in the SNc after LAC lesion, that was significantly decreased in xCT$^{-/-}$ mice compared to xCT$^{+/+}$ littermates, 3w post surgery (B). Similarly, LAC-induced loss of striatal DA content was significantly decreased in xCT$^{-/-}$ mice compared to xCT$^{+/+}$ littermates, 3w post surgery (A). Data are presented as percentage TH+ profiles or DA content compared to the intact side (mean ± SEM), and analyzed using 2-way ANOVA, followed by Bonferroni *post-hoc* tests (left panel), or Mann-Whitney U test (right panel). left panel: ***p<0.001 (2-way ANOVA), $^{###}$p<0.001 (Bonferroni *post*-hoc vs. sham); right panel: $^{###}$p<0.001 (Mann-Whitney U test). DA dopamine, LAC lactacystin, TH tyrosine hydroxylase, IHC immunohistochemistry SNc substantia nigra pars compacta.

The reason for this age-dependent protection needs to be elucidated in the coming months.

Next, we studied the involvement of system x_c^- in MPTP-induced parkinsonism, using the progressive, chronic MPTP model. Besides being progressive and chronic, this model has the advantage that no surgery is needed. MPTP is being delivered intraperitoneally instead of intracerebral. These experiments have been performed in collaboration with Prof. Dr. Charles Meshul (VA Medical Center, Portland, Oregon, USA) and are submitted to Neuroscience Letters (Bentea et al.). xCT expression levels have been measured in midbrain and striatum of MPTP-treated C57Bl/6 mice and we compared MPTP-induced nigrostriatal degeneration between $xCT^{-/-}$ and $xCT^{+/+}$ mice.

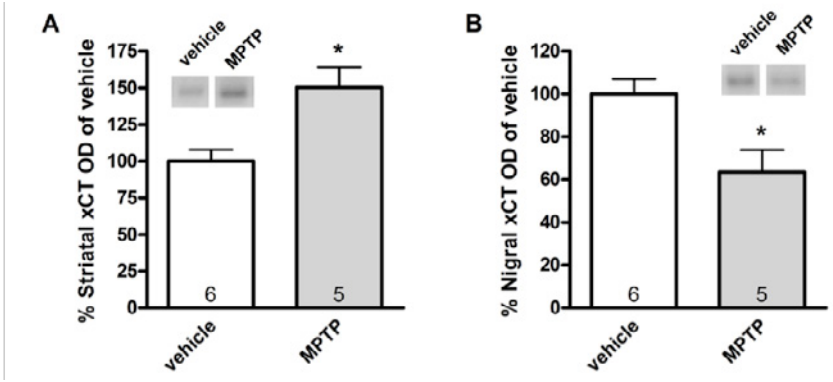

Figure 11: The effect of chronic and progressive administration of MPTP on xCT expression levels in the striatum and SN. (A) MPTP treatment led to an increase in xCT optical density in the striatum compared to vehicle-treated mice. (B) MPTP administration led to a decrease in xCT optical density in the SN compared to vehicle-treated mice. n = 6 vehicle group, n = 5 MPTP group. *P<0.05. Error bars represent SEM. Insets show representative xCT immunoreactive bands from each group (similar amounts of protein were loaded per lane, as measured using total protein stain).

Our results indicate that the expression of xCT undergoes region-specific changes in MPTP-treated mice, with increased expression in the striatum (fig. 11A), and decreased expression in the SN (fig. 11B). Furthermore, mice lacking xCT were equally sensitive to the neurotoxic effects of MPTP, as they demonstrate similar decreases in striatal dopamine content, striatal tyrosine hydroxylase (TH) expression, nigral TH immunopositive neurons and forelimb grip strength, five weeks after commencing MPTP treatment. Altogether, our data indicate that progressive lesioning with MPTP induces striatal and nigral dysregulation of system x_c^-. However, loss of system x_c^- does not affect MPTP-induced nigral dopaminergic neurodegeneration and motor impairment in mice.

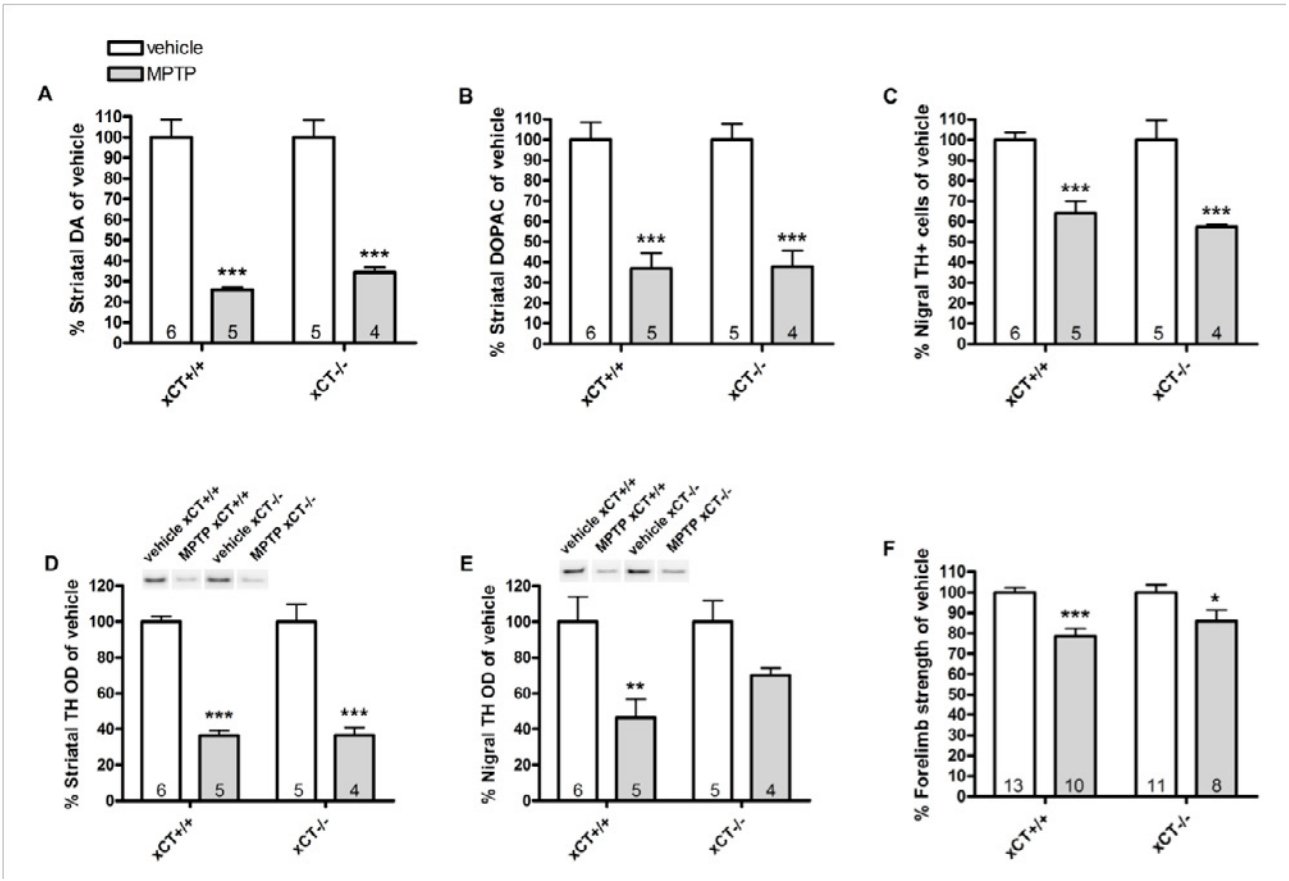

Figure 12: The effect of chronic and progressive administration of MPTP on the pathological and behavioral features in xCT$^{-/-}$ mice compared to xCT$^{+/+}$ littermates. (A and B) MPTP administration led to a similar loss of total striatal DA and DOPAC levels when compared to the vehicle group of each corresponding genotype. (C) TH immunohistochemistry revealed that MPTP induced a similar loss of nigral TH+ neurons in the two genotypes. (D and E) TH immunoblotting revealed a similar loss of striatal and nigral TH expression after MPTP in xCT$^{-/-}$ mice and xCT$^{+/+}$ littermates. n = 6 vehicle xCT$^{+/+}$ group, n = 5 MPTP xCT$^{+/+}$ group, n = 5 vehicle xCT$^{-/-}$ group, n = 4 MPTP xCT$^{-/-}$ group. (F) MPTP administration led to a similar loss of forelimb grip strength in xCT$^{-/-}$ mice and xCT$^{+/+}$ littermates. n = 13 vehicle xCT$^{+/+}$ group, n = 11 MPTP xCT$^{+/+}$ group, n = 10 vehicle xCT$^{-/-}$ group, n = 8 MPTP xCT$^{-/-}$ group. *P<0.05, **P<0.01, ***P<0.001 versus corresponding vehicle group. Error bars represent SEM. Insets show representative TH immunoreactive bands from each group (similar amounts of protein were loaded per lane, as measured using total protein stain).

3.3. For specific aim 3, we will investigate whether a local and timed downregulation (or inhibition) of system x_c^- can have the same protective effects as observed in animals that are born with a total loss of system x_c^-.

These experiments have not been initiated yet.

3.4. For specific aim 4, we investigated in detail anxiety and depressive-like behavior in non-PD xCT$^{-/-}$ mice compared to xCT$^{+/+}$ littermates. Moreover, the effect of loss of system x_c^- on anxiety and depressive-like behavior in PD mice will be evaluated.

We have completed a very thorough phenotyping study of the xCT$^{-/-}$ vs. xCT$^{+/+}$ mice. These results are currently under revision at Progress in Neuropsychopharmacology & Biological Psychiatry (Bentea, Demuyser et al.).

We phenotyped adult and aged system x_c^- deficient mice in a battery of tests for anxiety- and depressive-like behavior (open field, light/dark test, elevated plus maze, novelty suppressed feeding, forced swim test, tail suspension test). Concomitantly, we evaluated the sensorimotor function of system x_c^- deficient mice, using motor and sensorimotor based tests (rotarod, adhesive removal test, nest building test). Our results indicate that loss of system x_c^- does not affect motor or sensorimotor function, in either adult or

aged mice, in none of the paradigms investigated (fig. 13, 14). On the other hand, in the open field and light/dark tests, and forced swim and tail suspension tests respectively, we could observe significant anxiolytic (fig. 15) and antidepressive-like (fig. 16) effects in system x_c^- deficient mice that in certain cases (light/dark, forced swim) were age-dependent. These findings indicate that, under physiological conditions, nonvesicular glutamate release via system x_c^- mediates aspects of higher brain function related to anxiety and depression, but does not influence sensorimotor function. As such, modulation of system x_c^- might constitute the basis of innovative interventions in mood disorders.

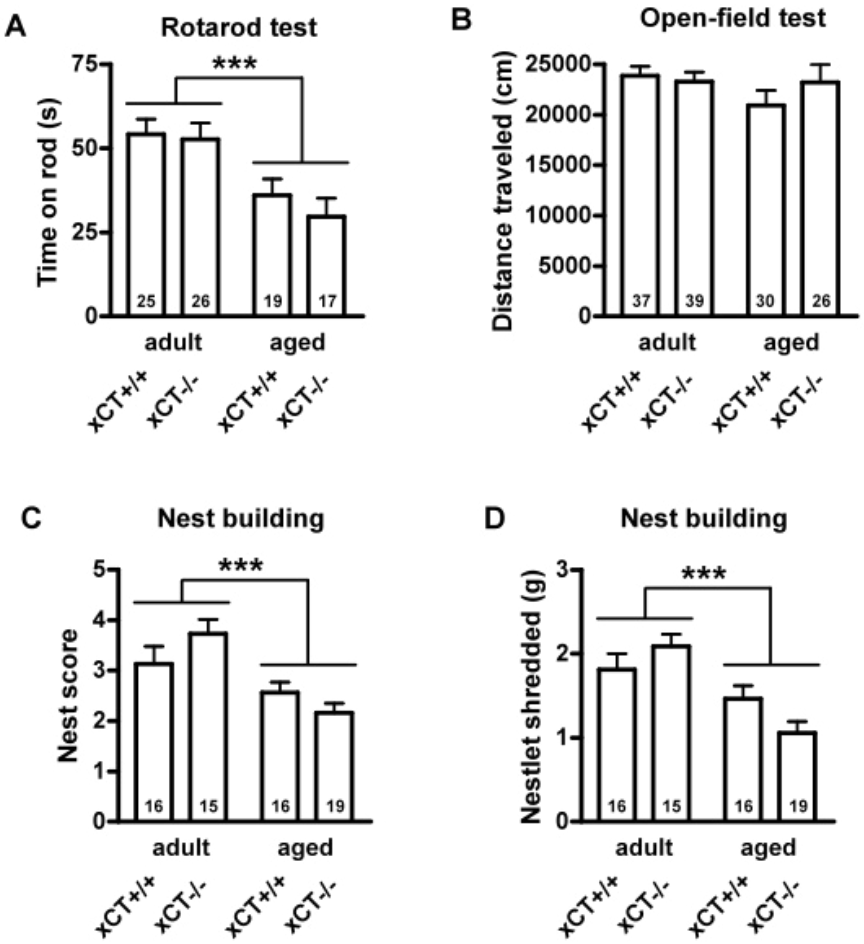

Figure 13: Motor behavior. Loss of system x_c^- does not affect motor coordination and balance as assessed using an accelerating rotarod protocol (A), nor does it change spontaneous motor activity and exploration as assessed in a 1 hour open field test (B), or fine motor skills and the capacity to build a nest (C, D). Data are presented as mean ± SEM. *** $p<0.001$ (2-way ANOVA), sample size indicated in figure.

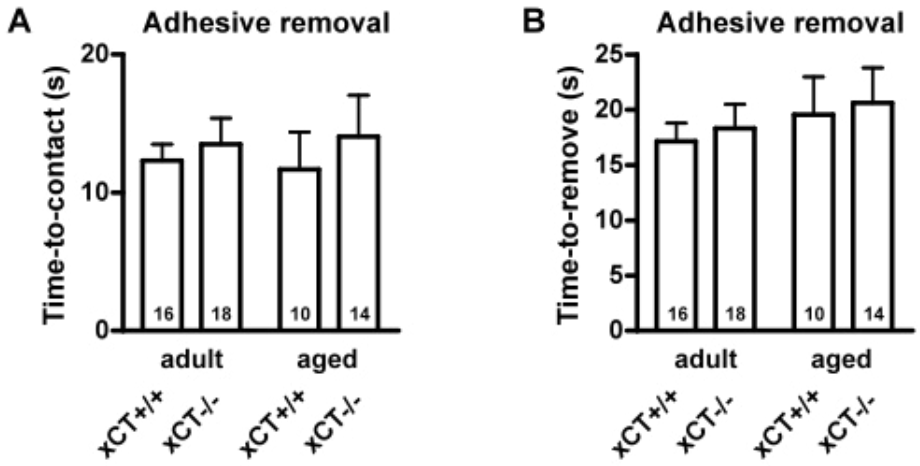

Figure 14: Sensorimotor behavior. Loss of system x_c^- does not affect sensorimotor function as evaluated in the adhesive removal test; $xCT^{-/-}$ mice demonstrate intact sensory function (time-to-contact, A), as well as intact fine motor skills (time-to-remove, B). Data are presented as mean ± SEM, sample size indicated in figure.

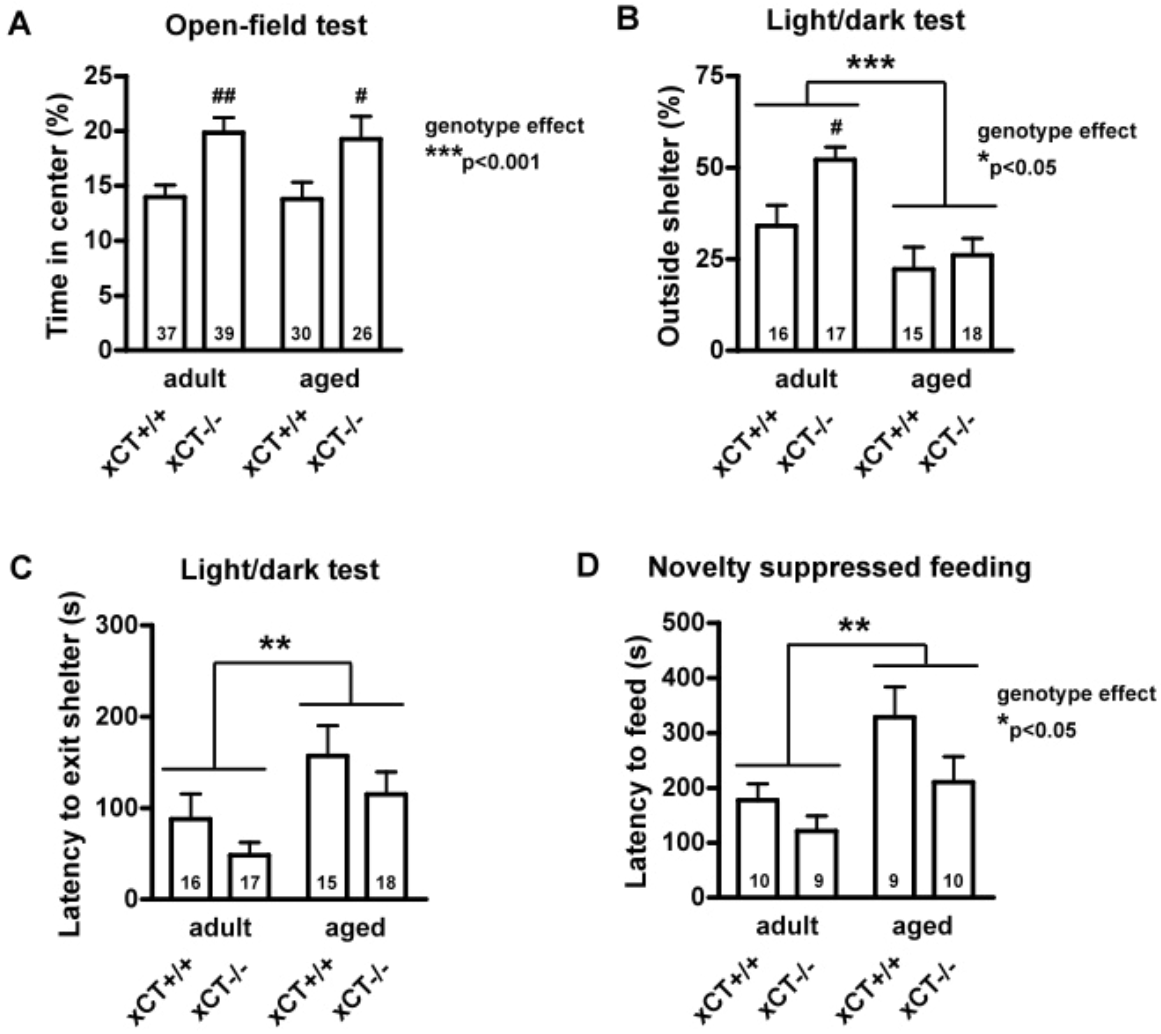

Figure 15: Anxiety-like behavior. Loss of system x_c^- leads to age-independent anxiolytic effects in the open field test (A), and age-dependent anxiolytic effects in the light/dark paradigm (B, C). Global anxiolytic effects could also be observed in the novelty suppressed feeding test (D). Data are presented as mean ± SEM *** p<0.001, ** p<0.01, * p<0.05 (2-way ANOVA), ## p<0.01, # p<0.05 (Bonferroni *post-hoc* versus age-matched $xCT^{+/+}$), sample size indicated in figure.

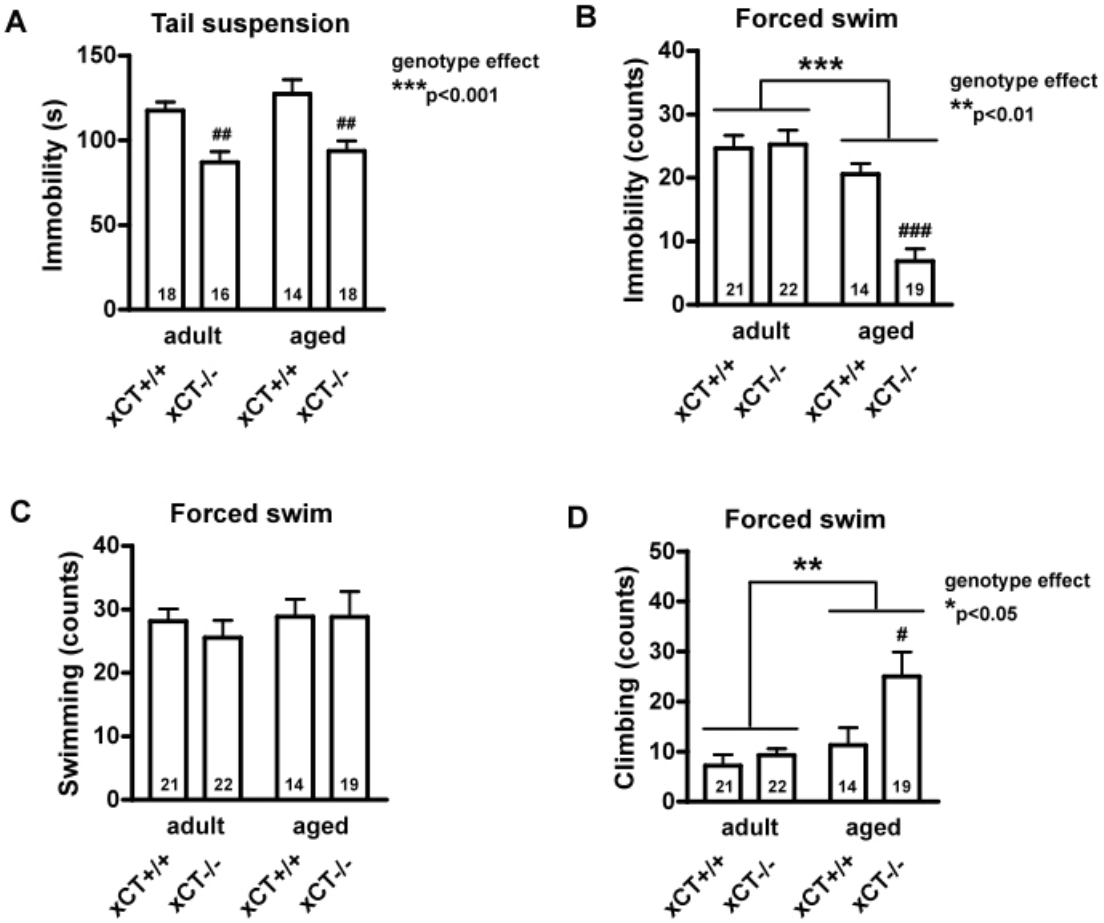

Figure 16: Depressive-like behavior. Loss of system x_c^- leads to age-independent antidepressive effects in the tail suspension test (A), and age-dependent antidepressive effects in the forced swim test (B), with increased climbing (D) but not swimming (C) behavior. Data are presented as mean ± SEM *** p<0.001, ** p<0.01, * p<0.05 (2-way ANOVA), ### p<0.001, ## p<0.01, # p<0.05 (Bonferroni *post-hoc* versus age-matched $xCT^{+/+}$), sample size indicated in figure.

The effect of loss of system x_c^- on anxiety- and depressive-like behavior in parkinsonian mice will be thoroughly investigated in the near future.

4. Publications obtained (or under revision/submitted) in the frame (or with the support) of the current GSKE project (2014-2016)

- El Arfani A, Bentea E, Aourz N, Ampe B, De Deurwaerdère P, Van Eeckhaut A, Massie A, Sarre S, Smolders I, Michotte Y (2014) NMDA receptor antagonism potentiates the L-DOPA-induced extracellular dopamine release in the subthalamic nucleus of hemi-parkinson rats. Neuropharmacology 85:198-205
- Bentea E*, Demuyser T*, Van Liefferinge J, Albertini G, Deneyer L, Nys J, Merckx E, Michotte Y, Sato H, Arckens L, Massie A*, Smolders I* (*equally contributing authors) Absence of system xc- in mice decreases anxiety and depressive-like behavior without affecting sensorimotor function or spatial vision. Progress in Neuro-Psychopharmacology & Biological Psychiatry, minor revision.
- Bentea E, Van Liefferinge J, Van der Perren A, El Arfani A, Albertini G, Demuyser T, Merckx E, Michotte Y, Smolders I, Baekelandt V, Massie A. Nigral proteasome inhibition in mice leads to parkinsonism including non-motor impairment and alpha-synuclein phosphorylation at Ser129. Frontiers in Behavioral Neuroscience, under revision.
- Bentea E*, Sconce MD*, Churchill MJ*, Van Liefferinge J, Sato H, Meshul CK, Massie A. MPTP-induced parkinsonism in mice alters striatal and nigral xCT expression but is unaffected by the genetic loss of xCT. Neuroscience Letters, submitted.

5. Publications obtained in the frame (or with the support) of the previous GSKE project (2011-2013)

- Van Liefferinge J, Jensen C, Albertini G, Bentea E, Demuyser T, Merckx E, Aronica E, Smolders I, Massie A. Altered vesicular glutamate transporter expression in human temporal lobe epilepsy with hippocampal sclerosis. Neuroscience Letters, minor revision.

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Deciphering the role of protein acetylation in primary ciliogenesis

Background and significance

By acting as a "sensor", the primary cilium has emerged as a key structure in a broad array of homeostatic and developmental processes. Although this immotile organelle has been discovered decades ago, little is known about its biology but accumulating evidence shows that mutations in cilia genes can lead to malformations underlying neurological or psychiatric disorders, as well as deafness and balance disorders [1]. The goal of the present project is to decipher whether and to what extend lysine acetylation of proteins controls primary ciliogenesis. In vertebrates, lysine acetylation sites are as conserved as those in phosphorylated proteins, suggesting a selective pressure to maintain this protein modification. Recent data indicate that acetylation occurs on thousands of proteins [2]. Although it was until recently exclusively associated with transcriptional activation (through neutralization of positive charges of core histone tails lysines[3]), there is now growing evidences to support lysine acetylation of a broad range of non-histone proteins[4-6]. This modification is promoted by lysine acetyl-transferases (KATs) and requires acetyl-CoA as acetyl donor. It is believed that lysine acetylation regulates activity, localization, specific interaction as well as stability/degradation of proteins, therefore controlling a variety of cellular processes such as apoptosis, proliferation and differentiation[7]. Recent studies suggest that acetylation of cytoplasmic substrates contributes to brain development[8, 9] and, that disruption of this process is associated with various progressive neurological disorders[10, 11]. Elongator deficiency in humans causes Familial dysautonomia, an autosomal recessive orphan neurodevelopmental and neurodegenerative disease, that mostly target neurons from the autonomic and sensory system[12, 13]. Elongator comprises six subunits (Elp1-6). Elp1 is a scaffold subunit required for functional assembly of Elongator and Elp3 acts as DNA demethylase[14] and a KAT. This multiprotein complex promotes Nε-acetylation of histone H3 lysines[15], thereby regulating elongation of some RNA messengers. However, the complex is mostly expressed in the cytoplasm where it controls acetylation of targets such as alpha-tubulin[8, 16] and Bruchpilot [17]. In addition, Elongator activity in yeast, worms and plants is mostly associated with its role in modifying wobble uridines in selected tRNAs. We recently uncovered the functional expression of Elongator complex in the developing cerebral cortex[8]. We showed that acetylation of α-tubulin in microtubules contributes to migration and differentiation of cortical projection neurons[8] and our preliminary data support a novel function of Elongator in primary ciliogenesis in radial glia progenitors of the cortex or the perinatal subventricular zone. Our aim is to uncover the role of protein acetylation in primary ciliogenesis by assessing regulation of Elongator acetylome as an entry point.

1. Assessing Elongator expression at primary cilium of radial glia progenitors

We are studying Elongator functions by coupling genetic, histological and biochemical approaches. Immunolabelings performed on hTERT-RPE1 cells uncovered Elp1 and Elp3 expression at PCM/basal body and/or transition zone. Similar results were obtained on perinatal radial glia cells *in vivo*. We crossed FoxG1:Cre mice [18] with Elp3 lox/lox mice to conditionally delete Elp3 from telencephalon progenitors (Elp3 cKO mouse), including radial glia cells. Our preliminary results showed that ACIII-positive primary cilia were significantly shorter in Elp3 cKO radial glia cells as compared to control, suggesting a role for Elongator in primary ciliogenesis. Ultrastructure analyses by electronic microscopy (SEM and TEM) confirmed shortening of the primary cilium upon Elp3 depletion in radial glia cells. Comparable data have recently been obtained in hTERT-RPE1 cells depleted of Elp3 by siRNA transfection. We are currently assessing the functional impact of cilium shortening on its ability to transduce molecular signals such as sonic hedgehog (shh) signalling.

Dorsal radial glia (also named apical progenitors or APs) showed primary cilium defects in Elp3cKO E12.5 embryos as compared to control. Both immunohistochemical labelings and SEM micrographs supported a shortening of the primary cilium in APs. Additional immunolabelings will be performed to assess the compartmentalized expression of Elp1 or Elp3 at primary cilium complex: primary cilium (colocalisation with ACIII and acetylated-α-tubulin, sstr3, MCHR1)[19], transition zone (colocalisation with Tmem216[20] or Cep290[21]), basal body (colocalisation with γ-tubulin and ninein for mother centriole[22]), and PCM (pericentrin[23]).

The AP cell cycle is coordinated with the interkinetic nuclear movement (IKNM). We will assess whether expression of Elongator subunits is dynamically regulated within primary cilium complex as cell undergo IKNM and progress through cell cycle. Pregnant dams will be injected with BrdU before embryo harvesting (E13.5) to label APs at specific phases of the cell cycle. The cell cycle length is about 14-16 hours at E14.5, thus 30 minutes BrdU pulse-labeling will label S-phase, 4 hours for G2/M, and 10 hours for early-to mid G1 phase. Immunostainings with anti-pHH3 will label M phase. We will couple confocal analyses of primary cilium proteins in Pax6/Sox2-positive APs with pHH3 or BrdU immunolabelings to assess the primary cilium complex expression of Elp1/Elp3 with respect to IKNM and cell cycle progression.

1.1. Assessing primary cilium-related phenotypes upon depletion of Elongator in radial glia progenitors

The size and thickness of telencephalon vesicles were reduced in E14.5 Elp3 cKO embryos, as compared to control. The microcephaly was not associated with increased cell death or cell cycle length modification. However, we noticed a precocious neuronal differentiation at the onset of corticogenesis (E12.5) in Elp3 cKO, as compared to control followed by loss of neurons that populate all cortical layers at later stages. While sox2-positive APs remained unaffected, the population of intermediate progenitors (IPs) generated by APs was reduced. Our preliminary results suggest that Elongator controls the proliferative behaviour of APs. We recently confirmed this hypothesis by showing that an acute depletion of Elp3 by in utero electroporation of Elp3lox/lox embryos with Cre-expressing vectors lead to increased generation of Tbr1-expressing neurons at the expense of Tbr2-expressing IPs without affecting the Pax6-positive cell population.

Since primary cilium has been shown to promote differentiative division of APs in another context[24], we are currently analizing whether AP primary ciliogenesis defects of Elp3 cKO may account for reduction of IPs thereby affecting the net production of cortical neurons. Cortical neurogenesis mostly depends on generation and amplification of IPs. These cells self-renew or generate two neurons while APs only generate a single neuron by round of division. We postulated that lack of Elp3 lead to reduced generation of IPs at the expense of neurons during differentiative division, thus reducing the overall number of cortical neurons in Elp3 cKO cortex. We will decipher whether any unbalanced regulation of cortical progenitors observed upon Elp3 depletion may be linked to primary ciliogenesis defects. We will also test whether this results from a loss of coordination between IKNM, cell cycle and primary cilium cycle progressions. In order to analyse the primary cilium upon depletion of Elp3 in APs, we will co-electroporate vectors expressing Cre-GFP (or GFP in controls) with Arl13b-RFP in E13.5 Elp3lox/lox embryos. This will consecutively remove Elp3 and highlight the primary cilium of APs. Pregnant dams will be injected with BrdU to analyse primary cilium with respect to cell cycle phase progression and electroporated embryos will be harvested the following day. We will couple primary cilium analyses (GFP) in Pax6/Sox2-positive AP with pHH3 and BrdU immunolabelings to assess primary cilium morphology with respect to both IKNM (soma positioning) and cell cycle progression. We will further perform real-time imaging[25] to monitor IKNM after cre-induced conditional removal of Elp3. For this purpose embryos will be harvested one day after electroporation for organotypic brain slice cultures, which will be further imaged (every 20 minutes for 16h[26]after 6 hours of recovery). Rescue experiments

will include conditional expression of centriole-localized PACT[27]-Elp3 and PACT-Elp3deltaHAT (control) in APs.

1.2. Identification of Elongator's targets in cortical primary ciliogenesis

Proteomic data revealed the existence of several proteins that may be acetylated by Elongator, among which some are known to control primary ciliogenesis. Although our current understanding of the contribution of protein acetylation to primary ciliogenesis remains elusive, these preliminary results showed that Elongator is localized at basal body of various cell types where it may control ciliogenesis by promoting lysine acetylation of primary cilium core components or its regulators. This is further supported by the identification of Elongator subunits in public cilium proteome (www.cildb.cgm.cnrs-gif.fr). We will use complementary approaches to identify primary core cilium components (or its regulators) acetylated by Elongator that are required during the early steps of cortical neurogenesis.

Sequencing the Elp3-dependent acetylome in the developing cerebral cortex

We performed an Elp3-dependent acetylome (AcetylScan from Cell Signalling Technology, BIOKE, Belgium: twenty E14.5 brains for each genotype will be analyzed). We obtained a list of proteins (54 candidates) that are less acetylated upon Elp3 depletion. Since Elongator controls elongation of various transcripts, we will perform deep sequencing analyses on cerebral cortical extracts from WT (Elp3lox/lox) and Elp3 cKO (Elp3lox/lox;FoxG1 :Cre) to identify and further discard transcriptional targets in order to focus on direct Elongator acetylation targets.

Validation of Elp3-acetylation targets in vitro and in vivo

The expression pattern of selected candidates in WT and Elp3cKO E12.5 to E16.5 cortices will be analysed by immunohistochemistry in order to check whether they are expressed at primary cilium complex of APs and IPs. We will further perform immunoprecipitations with anti acetyl-lysine antibodies[8] followed by immunoblots. This will validate our screen and confirm the *in vivo* acetylation status of candidates that show the most relevant expression pattern regarding primary ciliogenesis of cortical progenitors. If no commercial antibodies are available, we will subcontract the production of rabbit polyclonal ab against Elp3 targets (Phoenix France S.A.S.). The specific activity of Elp3 will be tested *in vitro* by performing acetylation assays with recombinant proteins, as previously described[8].

Identifying Elp3-acetylated proteins that contribute to primary ciliogenesis in cortical progenitors

The most relevant acetylation targets of Elp3 will be functionally analysed *in vivo* to assess whether their (de)acetylation contributes to primary ciliogenesis in cerebral cortical neurogenesis. To tackle this issue, endogenous candidate proteins will be swapped for corresponding acetyl-mutant (K-to-R mutants). This will be performed by *in vivo* co-electroporation of shRNA plasmids that target the candidate messengers together with plasmids coding for acetyl-mutants that are furthermore refractory to shRNA silencing (silent mutations) into cortical progenitors[8, 28]. We will further assess whether (de)acetylation of the candidate is critical for primary ciliogenesis by using acetylmimic mutant candidates (K-to-Q) in rescue experiments of Cre-electroporated Elp3lox/lox. We will monitor primary ciliogenesis in APs and IPs, balanced of proliferative and differentiative division of APs, IP specification and delamination upon conditional Elp3 depletion, as mentioned above.

2. Deciphering how Elongator is required for the formation of kinocilium and the development of the inner ear

Inner ear hair cells convert mechanical stimuli of sound, gravity and head movements into electrical signals. Each hair cell contains at the apical surface its mechanically sensitive organelle, the hair bundle consisting of actin-filled stereocilia and, at least during development, a tubulin-based primary cilium,

known as the kinocilium. In auditory hair cells of mammals and birds, kinocilia regress soon after the onset of mechano-transduction, whereas vestibular hair cells maintain kinocilia throughout maturity. Kinocilia of all the hair cells of the inner ear are polarized in a coordinated manner. The kinocilium is essential in sculpturing the inner ear and the polar hair bundles via the planar cell polarity (PCP) signaling pathway. Indeed, genetic loss or underdevelopment of the kinocilium led to PCP phenotype including misoriented hair bundles and a shorter and wider cochlear duct [29, 30].
Our preliminary results suggest that in the absence of Elp3 the kinocilium is shorter and PCP defects are present in the inner ear (figure 3). In this project, we will therefore test our hypothesis that genes - such as Elp3 - required for the formation of kinocilia is involved in PCP regulation at multiple steps for normal morphogenesis of the inner ear both in the vestibular and cochlear portions. We will first characterize ciliogenesis in the developing inner ear to establish developmental stages and molecular markers for functional analysis of ciliary genes. In a second time, we will study the role of Elp3 in inner ear ciliogenesis and PCP.

Characterization of ciliogenesis in the developing inner ear
Much of the fundamental knowledge about kinocilia is incomplete. In the absence of a reference structure of vestibular and cochlear kinocilium, studies on molecular mechanisms of ciliary functions are unattainable. The kinocilium is an enigmatic structure for two reasons: first, it is still unknown if it is a motile or non-motile cilium and second, it is a permanent structure in the vestibular portion of the inner ear, while it disappears around postnatal day 12 in the mouse cochlea. Therefore, in this part of our project we will proceed to a detailed characterisation of the auditory and vestibular kinocilia. Our preliminary results showed that ACIII does not seems to be present in cochlear and vestibular kinocilia. On the contrary, ARL13bis present in the axoneme. Other labelings are on going to characterize further the molecular composition of the kinocilium.
To further characterize these molecular differences, as compare to “classical” primary cilia, we will isolate kinocilia from the UB-OC1 cell line, derived from the developing inner ear of the immortomouse[31] by using a calcium-shock method as previously described[32] and analyse the resulting protein extract by Gel-Free Quantitative Proteomics (GIGA-Protemics platform, ULg). We will compare the obtained list of proteins with other systematic studies of cilia[33] and confirmed the new kinocilium proteins by immunohistochemistry. In parallel, we will examine the ultrastructure composition of kinocilia by transmission electron microscopy to decipher microtubule arrangements (i.e. 9+0 or 9+2) in axonemes of vestibular and cochlear kinocilium. Finally, we will study if dynein arms - conferring motility - are present on the peripheral microtubule doublets. We will also look at myosin VIIA and myosin Ic expression in the kinocilium. If we confirm their presence, as previously described [34, 35], we will study their role in the kinocilium. As no actin is present, one hypothesis should be that myosinVIIA or Ic participates to transport in kinocilium as motor proteins. We will try to identify microtubule binding domains in myosin VIIAor Ic sequence and if such domains are present, we will perform microtubule-binding assays as previously described[36] on full-length myosin VIIA or Ic and on mutated forms within the putative microtubule binding domain. This study will be the first step in a continuum of research to systematically characterise kinocilium in order to understand the roles of various ciliary proteins or their complexes.

Assessing PCP and kinocilium phenotype in the inner ear of Elp3 mutant mice
Preliminary results showed that Elp3 invalidation leads to shortening of kinocilium and PCP defects especially misorientation of stereociliary bundles and formation of truncated cochlear duct. To further analyse kinocilium defects, we will test whether Elp3 is required for polarization of the basal body, which tethers the ciliary axoneme to the cell and organises cytoskeleton. Cochlear and vestibular whole-mount explants isolated from WT and Elp3 cKO mice will be immunolabeled with specific markers of kinocilia identified in aim 4.1. If proteins are absent, they will constitute good candidate as Elp3 targets (see paragraph 4.3). Preliminary results showed that ARL13b is present in Elp3 cKO kinocilia (both in vestibular and cochlear portion).

To confirm PCP defects, membrane recruitment and asymetric localization of core PCP proteins will be studied. We will analyse by immunohistochemistry if Elp3 is required for membrane recruitment and asymetric position of dishevelled 2 (Dvl2), frizzled3 and vangl2, three core PCP proteins previously identified in the developing inner ear[37]. If some proteins are mispositioned, they will be good candidates as Elp3 targets (see paragraph 4.3). Preliminary results showed that vangl1, a protein normally asymmetrically localized in HCs, is present in the entire cytoplasm of HCs from Epl3 cKO mice, confirming a PCP defect in those mice.

Identification of Elp3 targets in kinocilium genesis

The most relevant candidate proteins obtained following SILAC and Acetylscan (paragraph 3.1) will be compared to the one identified in kinocilia (paragraph 4.1). To confirm the relevance of candidate proteins, we will first verify their acetylation using *in vitro* acetylation assays consisting in incubating recombinant Elp3 with the protein of interest and 3H-acetylcoA and after stopping the reaction analyze the reaction by fluorography as described[38]. Candidate protein acetylation status will be then studied in the cochlea and the vestibule (at key developmental stages) by immunoprecipitation with anti-acetyl lysine antibodies followed by immunoblot with antibody directed against the selected candidate. Finally, candidate proteins will be swap for acetylation-deficient mutants created by lysine to arginine conversion (K-to-R mutants). ShRNA plasmids that target the candidate messengers will be co-transfected together with plasmids coding for K-to-R mutants that are furthermore refractory to shRNA silencing (silent mutations) into E13.5 mouse inner ear explants or into UB-OC1 cell line. First, the subcellular localisation of wild type and K-to-R mutant candidate proteins will be studied by time-lapse videomicroscopy.

As Elp3 is known to acetylate histones and therefore facilitates transcription, we will study the transcriptome of Elp3 cKO and WT littermates by RNA-seq (GIGA genotranscriptomics, Ulg). RNA-seq libraries will be prepared, pooled and sequenced at 2x76bp (paired reads)/sequence using an Illumina Hiseq 2000 sequencer. Normalized expression counts will be used for differential expression analysis (DE-Seq, in collaboration with Wouter Coppieters, GIGA-genotranscriptomics platform). We will first focus on relevant molecules for kinocilium development (motor, PCP proteins). Relevant candidates will be confirmed by qPCR and immunohistofluorescence on WT and Elp3 cKO inner ears.

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4. Publications of the laboratory in 2014 supported by the F.M.R.E.

– Laguesse, S., Peyre, E., and **Nguyen, L**. :
Progenitor genealogy in the developing cerebral cortex
Cell Tissue Res (2014), 32(6): 1398-407 (I.F. 2013= 3.333)
– Volvert, M.-L.., Prévot, P.-P., Close, P., Laguesse, S., Pirotte, S., Hemphill, J., Rogister, F., Kruzy, N., Sacheli, R., Moonen, G., Deiters, A., Merkenschlager, M., Chariot, A., Malgrange, B., Godin, J., and **Nguyen, L.**,[CA]. :
MicroRNA targeting of CoREST controls polarization of migrating cortical neurons
Cell Rep (2014), 7(4): 1168-83 (I.F. 2013=7.207)
– Avila, A., Vidal, P.M., Tielens, S., Morelli, G., Laguesse, S., Harvey, J.H., Rigo, J.-M.*, and **Nguyen, L.***:
Glycine receptors control the generation of projection neurons in the developing cerebral cortex.
Cell Death Diff (2014), 21(11): 1696-708 (I.F. 2013= 8.385)

5. Other publications of the laboratory in 2014

– Genin, E.C., Caron, N., Vandenbosch, R.,**Nguyen, L**.,and Malgrange, B. :
Forkhead pathway in the control of adult neurogenesis
Stem Cells (2014), 32(6): 1398-407 (I.F. 2013= 7.701)

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Alteration of cholesterol turnover in Alzheimer disease: Molecular mechanisms and therapeutic applications.

Alzheimer disease (AD) is the current leading cause of dementia in the elderly. Brains of AD patients are characterized by the presence of senile plaques and neurofibrillary tangles, as diagnostic hallmarks, in addition to inflammation, synaptic and neuronal loss. The most prevalent hypothesis is the amyloid cascade hypothesis, stating that increased amyloid peptides (Aβ) induce a cascade of events including inflammation, neurofibrillary tangles, synaptic and neuronal loss, which thereby gives rise to the clinical symptoms in AD. This hypothesis is strongly supported by early onset inherited AD cases in which gene mutations increase production of more amyloidogenic Aβ, leading not only to development of senile plaques but also of neurofibrillary tangles. However, a successful clinical trial targeting Aβ is still lacking, despite many attempts. The lack of success of anti-amyloid therapies not only has challenged the amyloid cascade hypothesis but has highlighted the fact that combined therapies should be considered aiming not only at eliminating Aβ but also at regulating dyshomeostases leading to synaptic dysfunction.

The project that we have introduced at the FMRE was based on previous data from the literature indicating that perturbation of lipid metabolism favors progression of AD, in which processing of the Amyloid Precursor Protein (APP) has important implications. APP cleavage is tightly regulated by cholesterol and APP fragments regulate lipid homeostasis.

From these data, we decided to investigate whether up or down regulation of full-length APP expression affected neuronal lipid metabolism. Expression of APP decreased HMG-CoA reductase (HMGCR)-mediated cholesterol biosynthesis and SREBP mRNA levels, while its down regulation had opposite effects. APP and SREBP1 co-immunoprecipitated and co-localized in the Golgi. This interaction prevented Site-2 protease-mediated processing of SREBP1, leading to inhibition of transcription of its target genes. Although neuronal expression of APP strongly inhibited cholesterol biosynthesis, neuronal cholesterol content was not affected. Neurons are the only cells in the brain expressing 24-hydroxylase needed for hydroxylation of cholesterol into 14-S-hydroxycholesterol. Neuronal expression of APP decreased both HMGCR and cholesterol 24-hydroxylase mRNA levels, leaving neuronal cholesterol constant, but dramatically decreasing neuronal cholesterol turnover. Inhibition of cholesterol turnover completely inhibited neuronal activity, which was rescued by geranylgeraniol, generated in the mevalonate pathway. Altogether, these data indicate that APP controls cholesterol turnover needed for neuronal activity.

These results were published in EMBO Molecular Medicine (Pierrot et al. 2013), and we were invited by Medecine /Science to write a short paper in 2014 summarizing these data (Pierrot et al. 2014).

In AD patients, accumulation of cholesterol was observed in post mortem brain (Lazar et al. 2013), while a decrease in 14-S-hydroxycholesterol plasmatic concentration was measured in the late phase of the disease (Lutjohann et al. 2000). These results argue for a decreased cholesterol turnover in the late phase of AD. Since we demonstrated that cholesterol turnover is needed for neuronal activity, we reasoned that stimulation of cholesterol turnover should increase neuronal activity *in vitro* and synaptic plasticity in *in vivo* models of AD.

LXR/RXR nuclear receptors control transcription of genes involved in cellular uptake, transport, and efflux of cholesterol and thereby regulate neuronal cholesterol homeostasis, which is essential for basic synaptic function, plasticity and behavior. The beneficial effects of LXR/RXR Agonists (LRAs) on synaptic and cognitive functions were attributed to their ability to increase cholesterol efflux for proper lipidation

of apolipoprotein E (ApoE). We demonstrated however that in mouse brain, increased production of lipidated ApoE did not allow LRAs to improve synaptic plasticity. On the contrary, LRAs improved neuronal activity in cultured neurons in which cholesterol efflux was compensated by an HMG-CoA reductase (HMGCR)-mediated biosynthesis of cholesterol. Such a compensatory mechanism was also observed in 5xFAD mice, a mouse model of AD, in which LRAs rescued synaptic plasticity and cognitive deficits. We conclude that LRAs improve synaptic plasticity and cognition only when induced cholesterol efflux is compensated by HMGCR-mediated biosynthesis of cholesterol.

These results are under review in Nature Communication.

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  LXR/RXR nuclear receptors agonists improve synaptic plasticity by induction of HMG-CoA reductase-mediated biosynthesis of cholesterol.
  Nat Comm, under review.

En 2014, le financement de la FMRE a permis de prendre en charge le salaire d'une technicienne du 1/7/2014 au 31/12/2014, et de couvrir des frais de fonctionnement.

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Parkinson's disease revisited. A new vision of basal ganglia functions in the context of the Parkinson's disease

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, caused by a neuronal loss in the Substantia Nigra, pars compacta (SNc), which leads to a depletion of dopamine in the striatum. PD is characterized by a well-known triad of symptoms, namely bradykinesia, rigidity and tremor at rest, although non-motor symptoms, often under-appreciated, are increasingly being recognized. However, despite decades of intense investigation, the precise role of the basal ganglia (BG) to motor control and their contribution to PD symptoms remain debated.
Indeed, the exact link between the dopamine depletion in the striatum and motor symptoms of PD remains puzzling. Recently, it has been suggested that the loss of dopaminergic neurons in the SNc, instead of leading to a motor disorder *per se*, affects the reward and decision-making processes. More precisely, the main cause of bradykinesia could be the inability to adjust the level of effort invested in movements due to an inappropriate estimate of their cost/benefit ratio. These observations have led to an interesting hypothesis about the role of BG in controlling the movement "vigor" according to motivational factors; this is the first topic we planned to address in this research project.
Amongst the motor functions assigned to the BG, a significant one is the storage and recall of overlearned sequential skills. However, this view has been questioned by recent studies in non-human primates and growing evidence suggests that, instead, the BG play a causal role in the learning process of those skills. Interestingly, it has been shown recently that the putamen, a part of the striatum, is recruited during chunking, a mechanism which permits to group discrete movements as a single complex action. To determine the neural correlates of chunking, and the contribution of BG to chunking in particular, is the second main question we will address in this project.

1. Physiopathology of bradykinesia: role of BG in movement vigor.

One of our working hypotheses is that one function of the BG is the ability to adjust the level of effort invested in actions as a function of the expected outcome value, and this impairment leads to bradykinesia, a cardinal feature in PD. To address this issue we ran several experiments aiming at determining the role of the BG in effort and reward coding, in order to understand better the physiopathology of bradykinesia. Here are the summary of these different studies.

1.1. The Human Subthalamic Nucleus encodes Reward and Effort utility during cost-benefit decision-making.

Adaptive behavior entails the capacity to select actions as a function of their energy cost and expected value. Animal and human studies indicate that this function relies on a network of cortical areas linked to ventral striatum, but strong evidence also points to the involvement of other basal ganglia structures Given its known role in motivational processes in animal models, and its privileged position within the basal ganglia, we hypothesized that the Subthalamic Nucleus (STN) could be an important actor in the effort-based decision making circuit. However, this putative function appears at odds with the recent evidence suggesting that STN is important for adjusting response speed in conflict situations. Here we directly tested these contrasting hypotheses and found that neuronal activity in the human STN encodes both the subjective value of reward and the subjective cost of effort, rather than signaling decision conflict. We recorded local field potentials in the STN of patients with Parkinson's disease during an effort-based decision task. We found strong, low-frequency neuronal responses to reward

and effort information delivery in the STN, which was proportional to reward and effort utility and did not depend on the effector used to provide the response, nor, importantly, on the level of decision conflict. These utility signals were disrupted under conditions of dopamine withdrawal. Our findings demonstrate that low-frequency responses in STN encode the value of reward and the cost of effort during cost-benefit computation. They could reflect a resource-allocation signal, mobilizing attentional and/or physical effort in the face of motivating cues or demanding situations.

1.2. Tonic dopamine level modulates response vigor independently of opportunity cost

Dopamine signalling can be separated in 2 components: the phasic response, often thought to represent the reward prediction error, and the tonic level, known to affect response vigor. In a recent computational model, tonic dopamine concentration was proposed to represent opportunity cost, i.e. the average value of all the forfeited actions. According to this view, increased tonic dopamine would lead to higher response vigor because it allows to decrease the time spent in executing the chosen action, leaving more time for the other valuable options.

Here we investigated this hypothesis by testing 19 healthy subjects on two effort-based decision making tasks. In task 1, participants could choose to execute power grip efforts of different intensities for varied monetary rewards. Crucially, the level of force they exerted during a given trial influenced the time they spent executing the effort, while the reward they earned was kept fixed. In task 2, in contrast, the effort duration was imposed and exerting more force allowed the subject to earn more reward instead of saving time. Participants performed these tasks in 2 sessions, following either 125 mg of levodopa or placebo administration.

We found that, opposite to the model predictions, levodopa administration caused an increase of the force exerted in task 2 only, in which increased force led to higher rewards but not to reduced effort duration. This suggests that tonic dopamine does not signal opportunity cost but rather influences the incentive salience, or motivational value, of the reward.

1.3. Dopamine modulation of reward- and effort-based decision making circuits in humans

Cost-benefit computations are known to rely crucially on tonic dopamine levels. Dopamine depletion in rats leads to shifts in behavior away from effortful choices. In addition, lesions of Nucleus Accumbens in ventral basal ganglia or the dorsal part of Anterior Cingulate cortex lead to comparable effects. Similarly, in humans, tasks requiring to balance reward with effort are associated with BOLD activations in the same brain structures, and dopamine increases lead to behavioral shifts in favor of effortful choices. However, it remains unclear how these structures interact with each other and how dopamine affects their functioning.

Here we addressed this issue by measuring brain activity by means of functional magnetic resonance imaging during the performance of a reward- and effort-based decision making task and determining the effect of dopamine manipulations on these activations and on behavior. Participants (n=13) had to decide whether or not they wanted to execute an effort consisting in squeezing a hand dynamometer, as a function of the effort intensity and the reward proposed for this effort. They performed this task in a 3T MRI scanner. Dopamine manipulations followed a double blind protocol, using either placebo, levodopa 125mg or sulpiride 400 mg. The pills were administered so that maximal blood concentration would be reached during the task execution. Each subject performed the task three times, once for each dopamine manipulation.

Preliminary results indicate that dopamine increases led to augmented effort investment in the task and that, conversely, dopamine antagonism led to decreased effort. fMRI data showed that a circuit involving dopaminergic midbrain, Nucleus Accumbens and Anterior Cingulate Cortex reflected cost-benefit computations during the task and that the same circuit was modulated by dopaminergic manipulations. We also performed functional connectivity analyses that confirmed the existence of strong interactions between these structures during the task.

These initial findings confirm that a circuit involving dopaminergic midbrain, Nucleus Accumbens and Anterior Cingulate Cortex play a central role in comparing effort costs with reward in order to reach a decision about whether or not to engage in an action. This circuit is strongly modulated by dopamine in a way that follows behavioral effects of dopamine manipulations.

2. Neural correlates of chunking: role of BG in motor learning.

Another central question of this research project is the possible role played by the BG in motor chunking, possibly through a thalamo-cortical loop involving Broca's area. This function might explain the deficits in sequence learning reported in PD patients and to provide a cohesive view to clarify the BG contribution to motor learning. First we developed a new method to analyse chunking strategies, which are difficult to evaluate and quantify. We have also investigated the contribution of Broca's area in chunking and the possible relationship between chunking and working memory. Here are the abstracts of these different studies.

2.1. New method to identify chunks: Is there still evidence for concatenation?

Chunking is a well-known cognitive mechanism used in our daily life in order to optimize efficiently the usage of working memory. It consists in clustering a long sequence of items in small set, termed chunks, with measurable behavioral effect, i.e. reaction times. Despite the topic is covered by a considerable amount of literature, an efficient method to identify and analyze chunks is still lacking. Here we developed a simple and computationally easy to implement method in order to quantify chunking strategies, allowing us to report not only the number of chunks, but their position. The algorithm is based on a ranking system combined with an index that quantifies the reliability of the results obtained. We tested our algorithm on ad hoc simulated data in order to validate it and quantify its accuracy, varying the level of noise in the data. Furthermore, our algorithm was applied to reaction time series acquired in distinct experiments, from three different dataset. Surprisingly, our analysis failed in revealing any evidence in favor of a process known as concatenation, thus suggesting that the number of chunks and the strategy employed by the subject remain constant across the whole experiment. This finding partially matches few studies on the same topic, although it does not conform to other papers in the literature.

2.2. Disruption of Broca's area alters high-order chunking processing during perceptual sequence learning

Broca's area appears to be involved in many cognitive functions, and most particularly in language, syntax and action processing. Chunking, which consists in grouping successive elements of a sequence in order to facilitate their memorization, plays an important role in these various cognitive processes and demonstrating that a role of Broca's area in chunking could help explaining the apparent multiplicity of its functions. Recently, we have postulated that Broca's area might be involved in high-order chunking. Here, to test this hypothesis, we used off-line MRI-guided transcranial magnetic stimulation in healthy volunteers to disrupt the activity of the posterior part of Broca's area (left Brodmann area (BA) 44) just before subjects learned a perceptual sequence structured in distinct hierarchical levels. We found that disrupting left BA44 increased the processing time of stimuli representing the boundaries of high-order chunks.

The current study highlights the possible role of left BA44 in building up an effector-independent representation of higher-order events in structured sequences. This might clarify the contribution of Broca's area in processing hierarchical structures, a central mechanism in many cognitive systems, such as language and composite actions.

2.3. Chunking mediated improvement in sequence performance depends on working memory gating mechanism.

A powerful tool to circumvent the limits of working memory is information reorganization or recoding. Known as “chunking”, the ability to either meaningfully or rhythm-driven, to group information in working memory into sub-groups, named “chunks”, has been extensively studied. Although chunking has been shown to be an efficient strategy to increase the storage of information into working memory, it remains unclear whether chunking also improves sequence performance in terms of execution time. Here, we propose that the key factor of efficient sequence performance is the chunk stability. Thus, we introduce a chunk carryover index, which reflects how stable is the chunking pattern during the experiment. We also hypothesize that chunking sequential information and persistence of chunking strategy might involve dynamic gating of working memory in defining chunks boundaries in the continuous information flow as well as output gating of working memory, while selecting appropriate chunks for execution. Our finding that chunks carryover index correlates with degree of improvement in subjects performance shows that chunking, and the stability of chunking pattern in particular, play an important role in performance improvement. We also found a strong positive correlation between working memory updating task, often used to measure efficacy of working memory gating, and chunk carryover index, which suggest the dependency of chunking on working memory gating mechanisms.

2.4. Dissociable factors of improvement in motor sequence performance depending on conscious intention.

Our daily life would be impossible without the ability to perform sequential motor actions. While some of motor sequences are learnt with explicit intention, the others are acquired implicitly, without our conscious awareness. Despite of the amount of research done on motor learning, many aspects of the difference between explicit and implicit motor sequence learning conditions remain unclear. In order to address this question, we separated subjects either into implicit or explicit group, and used enhanced sequence learning paradigm. Participants of the implicit group were not informed about the presence of the sequence, and did not report having notice any sequential structure after the experiment, while subjects of the explicit group were instructed to improve their performance by learning the sequence. All of the subjects performed sequence learning task using KINARM (kinesiological instrument for normal and altered reaching movements) End-Point robotic device that allowed us to obtain variety of movement parameters. Importantly, using this paradigm we were able to dissociate reaction time into movement time and onset time, with later being a good measure of possible anticipatory movements. We were also interested in how participants of both groups would use “chunking”, which denotes grouping of sequence items into subgroups and can affect subjects’ performance. We measured chunking with 2 indexes: how stable and how far from uniform distribution chunking strategy of the subject was. As we expected, explicit group improved their reaction times much more than implicit. Overall, as subjects of explicit group learned the sequence, most of their movements became anticipatory, so performance improvement was driven by decrease in onset time, with no change in movement time. On the other hand, subjects of implicit group improved their performance by means of decreasing movement time, while onset time did not change across the experiment. Interestingly, this difference cannot be attributed to chunking, as we found that both groups utilized chunking to the same extent. These results for the first time show differential mechanisms of performance gain in explicit and implicit motor sequence learning

Finally, it is worth mentioning that we have also published in 2014 an opinion paper on the possible role of the BG in spoken language (see reference 3.1).

3. Références

(*The publication's numbers refer to the Abstract's numbers mentioned in the main text)*

1.1. Zénon A., Yann Duclos, Romain Carron, Tatiana Witjas, Christelle Baunez, Jean Régis, Jean-Philippe Azulay, Peter Brown, Alexandre Eusebio.
The Human Subthalamic Nucleus encodes Reward and Effort utility during cost-benefit decision-making. *Submitted.*

1.2. Zénon A., Sophie Devesse, Etienne Olivier
Tonic dopamine level modulates response vigor independently of opportunity cost *Abstract presented at the PhD student days, IoNS, UCL, November 2014*.
Manuscript in preparation.

1.3. Zénon A., Etienne Olivier, Laurence Dricot
Dopamine modulation of reward- and effort-based decision making circuits in humans
Abstract presented at the Society for Neuroscience (SFN) meeting, Washington DC, November 2014.
Manuscript in preparation.

2.1. Alamia A., Oleg Solopchuk, Alexandre Zénon, Etienne Olivier
New method to identify chunks: Is there still evidence for concatenation?
Manuscript in preparation.

2.2. Alamia A, Violette Van Bever, Alessandro D'Ausilio, Luciano Fadiga, Etienne Olivier, Alexandre Zénon
Disruption of Broca's area alters high-order chunking processing during perceptual sequence learning. *Submitted.*

2.3. Solopchuk O., Andrea Alamia, Etienne Olivier, Alexandre Zénon
Chunking mediated improvement in sequence performance depends on working memory gating mechanism.
Abstract presented at the PhD student days, IoNS, UCL, November 2014
Manuscript in preparation.

2.4. Solopchuk O., Andrea Alamia, Etienne Olivier, Alexandre Zénon, Jean-Jacques Orban
Dissociable factors of improvement in motor sequence performance depending on conscious intention.
Manuscript in preparation.

3.1. Zénon A, Olivier E.
Contribution of the basal ganglia to spoken language: Is speech production like the other motor skills? *Behav Brain Sci. 2014 Dec;37(6):576*.

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The ephrin axon repellent system in amyotrophic lateral sclerosis

State of the art and summary of the research project

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder of motor neurons in adults, resulting in muscle atrophy and weakness. Its course is relentlessly progressive; the disease is fatal within three to five years after onset. There currently is no cure for ALS. Mutations in more than 10 different genes are known to cause the hereditary form of ALS: SOD1 and C9ORF72 mutations are the most prevalent ones, while mutations in TDP-43 and FUS are less frequent [1]. Mutant SOD1 transgenic mouse models faithfully reproduce the human disease and have been studied extensively over the last two decades [2]. Phenotypic variability of ALS is considerable, even in patients in whom the disease is caused by the same molecular abnormality. This indicates that factors, environmental or genetic in nature, modify the phenotypic expression of these diseases. It is important to identify such modifying factors, as they may represent targets for therapeutic intervention, in particular for the sporadic forms of neurodegenerative diseases, of which we do not know the cause.
In previous research, we identified one such factor, the ephrin receptor EphA4, as a modifying factor for ALS [3]. Genetic and pharmacological inhibition of EphA4 rescued the phenotype in a zebrafish model of ALS and increased survival in ALS rodent models. In ALS patients an inverse correlation was found between EphA4 expression and disease onset [3].
In order to facilitate a drug development strategy targeting the EphA4 pathway, this project intends to investigate the mechanism through which EphA4 affects motor neuron degeneration in ALS. This is being done by dissecting the EphA4 pathway and exploring the contribution of different biological aspects of the pathway in the motor neuron degeneration that occurs in ALS. This project also contemplates to target EphA4 pharmacologically.

Results

1. Mechanism of involvement of EphA4 in motor neuron degeneration

1.1. Identification of cell type mediating the effect of EphA4 on motor neuron degeneration

In order to discriminate which cell type mediates the effect previously observed of EphA4 on motor neuron degeneration, a triple transgenic mouse is being generated. This mouse, the Thy1-CreER::EphA4$^{lox/lox}$::SOD1^{G93A} mouse, expresses CreER (Cre recombinase-estrogen receptor fusion protein) in a subset of neurons among which the motor neurons, so that it will lack EphA4 in the motor neurons after treatment with tamoxifen. We are currently crossing the SOD1^{G93A} mice to an EphA4$^{lox/lox}$ mouse (JAX laboratories) and to a Thy1-CreER mouse (JAX), in order to obtain the triple transgenic mouse. As a control, another triple transgenic mouse is being generated, the CAGG-CreER::EphA4$^{lox/lox}$::SOD1^{G93A}. In these mouse models, when treated with tamoxifen, EphA4 will be deleted in all cell types. In the future, mice will be treated with tamoxifen at day 60 and disease parameters will be studied.

1.2. Signalling direction of the modifying effect of EphA4 in ALS

EphA4 and its ligands are transmembrane and membrane bound proteins. Bidirectional signalling occurs when the receptor and its ligands interact, resulting in forward signalling in the cell that bears the receptor and in reverse signalling in the cell that bears the ligand [4]. The EphA4^{eGFP} mouse (kindly provided by Prof. Rudiger Klein, Max Planck Institute Martinsried, Germany) is a mouse that has proofed to be a good strategy to discern between these two signalling directions [5, 6]. This mouse has a

knock-in replacement of the whole intracellular domain of EphA4 by eGFP and cannot trigger forward signalling. If the forward signalling is playing a role in ALS disease we expect similar effects in this transgenic mouse (EphA4^{eGFP}::SOD1^{G93A}) compared to those observed in the past, when EphA4 levels were lowered in the SOD1^{G93A} mouse (EphA4$^{+/-}$::SOD1^{G93A}) [3]. On the other hand if the beneficial effect of deleting EphA4 is mediated through a reduction in reversed signalling we would expect no difference in the clinical presentation of this mouse (EphA4^{eGFP}::SOD1^{G93A}) compared to the control (SOD1^{G93A}). The EphA4^{eGFP} mouse has been crossed with the SOD1^{G93A} mouse and clinical disease parameters have been studied. Neither disease onset nor disease survival is altered in EphA4^{eGFP}::SOD1^{G93A} mice compared to EphA4^{eGFP}::SOD1^{G93A} mice (Figure 1). We are currently quantifying the survival of motor neurons in the spinal cord of EphA4^{eGFP}::SOD1^{G93A} mice and the innervation of the neuromuscular junctions in these mice.

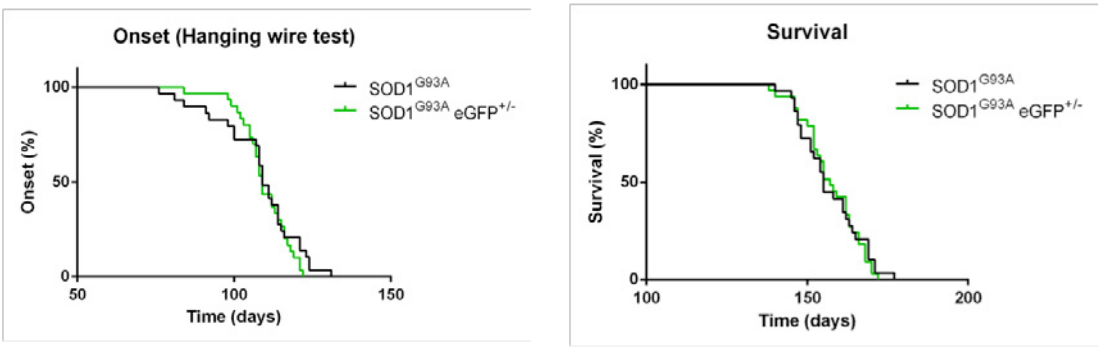

Figure 1. Forward signalling does not play a role in ALS as determined with the EphA4^{eGFP}mouse. Onset, which was determined by lack of performance on the hanging wire test (N=27-30), and survival were determined in EphA4^{eGFP}::SOD1^{G93A} and compared to SOD1^{G93A} mice (N=29-30). No difference was observed in onset (109 days in SOD1^{G93A} Epha4 eGFP$^{+/-}$ versus 109 days in SOD1^{G93A}) and neither in survival (156 days in SOD1^{G93A} Epha4 eGFP$^{+/-}$ versus 155 days in SOD1^{G93A}).

Additionally, in order to more robustly underscore the signalling direction involved in the effect of EphA4 on motor neuron degeneration in the SOD1^{G93A} model we are also crossbreeding the SOD1^{G93A} mice with a mouse in which the kinase domain of EphA4 has been inactivated by substituting a lysine in position 653 by methionine (K653M) generating a non-functional kinase EphA4: EphA4^{KD} (kindly provided by Prof. Rudiger Klein, Max Planck Institute Martinsried, Germany) [7].

1.3. Ligands for EphA4 in motor neuron degeneration

There are two major classes of ephrins and ephrin receptors, called A and B. In mammalians there are 5 types of ephrin-A (ephrin A1 to A5) and 3 types of ephrin-B (B1 to B3). Ephrin-As are GPI-anchored proteins while ephrin-Bs have a transmembrane domain. EphA4 is a promiscuous receptor that is able to bind most of ephrin-A and B [4]. Therefore, EphA4 could be an ALS disease modifier by interacting with almost any ephrin. The presence of the different ephrins in the spinal cord will be studied using qPCR. We will study symptomatic (age 60 days), early symptomatic (age 120 days) and late symptomatic (age 140 days) SOD1^{G93A} mice and age-matched wild-type SOD1 overexpressing (SOD1^{WT}) mice as controls. We will also identify the cell type in which the ligands are expressed using immunohistochemistry or in situ hybridization (since antibodies are not available for all ephrin ligands). SMI32 staining will be used to identify (motor) neurons, GFAP for astrocytes, Iba1 for microglial cells, and NG2 for oligodendrocyte precursor cells. We are currently collecting the samples at these various time points as indicated.
In preliminary experiments, we have already identified one ligand, ephrin-B2, to be abundantly upregulated in reactive astrocytes in ALS. To determine if an upregulation of ephrin-B2 in reactive astrocytes could be implicated in the pathophysiology of ALS, we are generating a triple transgenic mouse to specifically excise ephrin-B2 from astrocytes. We were kindly provided with a transgenic mouse, which expresses Cre driven by the connexin-30 (Cx-30) promoter, the Cx30-CreER mouse (Dr. J. Frisen, Karolinska Institute, Stockholm, Sweden). The breeding of the Cx30-CreER::ephrin-B2$^{flox/flox}$::SOD1^{G93A} is ongoing.

2. Therapeutic significance of the involvement of EphA4 in motor neuron degeneration

2.1. Use of KYL, an EphA4 antagonist

We have determined the toxicity of intracerebroventricular administration of the KYL peptide in the mouse, by studying motor performance of animals treated with 3 mM, 5mM and 10 mM per day. Only at a dose of 10 mM, the motor performance of mice on the rotarod was slightly (and statistically non-significantly) affected. At a concentration of 5 mM there was a risk of drug precipitation inside the pumps. Therefore we decided to lower the KYL peptide concentration to 3 mM and 1.5 mM. We are currently breeding a cohort of SOD1^{G93A} mice to perform a trial using the KYL peptide. We will use the KAL peptide as control. Outcome will be evaluated using clinical parameters and quantification of motor neuron degeneration (motor neuron counts and neuromuscular innervation).

2.2. Use of anti-EphA4 nanobodies

EphA4 inhibitors identified so far still have poor physicochemical properties and low pharmacodynamic and pharmacokinetic properties. They have very low stability, affinity or specificity for EphA4 [8]. Nanobodies are naturally occurring; heavy-chain only antibodies produced in *Camelidae*, with unique physicochemical and pharmacokinetic properties that make them match the requirements of many biomedical applications. Like conventional antibodies used in biomedicine they have a high specificity and affinity for their target as well as low inherent toxicity. In addition to that, they are very stable and small in size, which gives the opportunity to inhibit enzymes and readily access receptor clefts.
In collaboration with Dr. G. Hassan Zadeh Ghassabeh (Dept of Structural Biology, University of Brussels), we have developed 14 nanobodies, single-chain antibodies produced in *Camelidae*, directed against the extracellular domain of EphA4, using previously described methods [9].
We have determined the affinity of all 14 nanobodies for the mouse and human EphA4 (>98 % identity) and also for the human EphA4 extracellular domain by surface plasmon resonance (SPR, Biacore, Figure 2). At least 4 nanobodies have affinities which are 100x of the most specific known EphA4 antagonist, the KYL peptide.

A

	N-terminal EphA4 (human)			Human EphA4 – human Fc			Mouse EphA4 – human Fc			Model used
	Steady state	Kinetics analysis		Steady state	Kinetics analysis		Steady state	Kinetics analysis		
	K_D (M)	K_D (M)	R_{max} (RU)	K_D (M)	K_D (M)	R_{max} (RU)	K_D (M)	K_D (M)	R_{max} (RU)	
3EAR53	4.932×10^{-8}	4.574×10^{-8}	152	2.710×10^{-8}	2.26×10^{-8}	87%	2.700×10^{-8}	2.28×10^{-8}	170%	1:1
3EAR39	6.187×10^{-8}	5.656×10^{-8}	153	3.505×10^{-8}	3.013×10^{-8}	93%	3.183×10^{-8}	2.359×10^{-8}	100%	1:1
4EAR22	6.458×10^{-8}	5.410×10^{-8}	109	6.047×10^{-8}	4.708×10^{-8}	85%	1.171×10^{-7}	1.041×10^{-7}	87%	1:1
3EAR57	6.602×10^{-8}	5.752×10^{-8}	129	4.174×10^{-8}	$3,8311 \times 10^{-8}$	92%	4.170×10^{-8}	$3,7786 \times 10^{-8}$	93%	1:1
3EAR60	6.860×10^{-8}	3.696×10^{-8}	138	3.906×10^{-8}	3.958×10^{-8}	92%	2.194×10^{-8}	2.741×10^{-8}	97%	1:1
3EAR50	1.136×10^{-7}	8.733×10^{-8}	111	6.785×10^{-8}	6.241×10^{-8}	171%	9.046×10^{-8}	1.037×10^{-7}	56%	1:1/ Two state
3EAR31	3.047×10^{-7}	2.930×10^{-8}	98	2.012×10^{-7}	1.750×10^{-7}	167%	2.737×10^{-7}	2.585×10^{-7}	59%	Two state
4EAR28	3.763×10^{-7}	3.314×10^{-7}	73	2.299×10^{-8}	1.316×10^{-8}	97%	1.261×10^{-5}	5.288×10^{-8}	124%	1:1
4EAR19	3.869×10^{-8}	5.069×10^{-7}	39	1.027×10^{-8}	8.516×10^{-7}	78%	1.946×10^{-8}	1.143×10^{-8}	69%	1:1
4EAR90	/	8.371×10^{-8}	5095	2.012×10^{-7}	1.750×10^{-7}	167%	2.737×10^{-7}	2.585×10^{-7}	59%	1:1
4EAR34	/	/	/	2.106×10^{-5}	1.803×10^{-5}	91%	2.628×10^{-5}	2.690×10^{-5}	72%	Two state
4EAR47	/	/	/	Hardly any signal above background						/
4EAR71	/	/	/	Hardly any signal above background						/
3EAR16	/	/	/	Hardly any signal above background						/

Figure 2. EphA4 nanobody affinity to EphA4. Overview of dissociation constants of the different nanobodies determined with SPR.

Currently we are further analysing the specificity of the different nanobodies for EphA4 by determining the affinity of the different nanobodies for other Eph receptors.
In future experiments we will determine which nanobody is able to most potently block EphA4 phosphorylation upon stimulation with a ligand. The best nanobody will be selected based on the results of all these studies, and will be used for a clinical trial in mice.

2.3. Inhibition of the EphA4 signalling cascade: ROCK inhibitors

EphA4 stimulation results in growth cone collapse through activation of GTP-bound RhoA activity, which phosphorylates ROCK. ROCK phosphorylates LIMK, which in turn phosphorylates and thus inhibits cofilin, resulting in reorganization of the actin filamentous network. Fasudil, which is an effective ROCK inhibitor, stabilizes the cytoskeleton and it is in clinical use for vasospasm such as seen after subarachnoidal hemorrhage, for stroke and for pulmonary hypertension [10, 11]. Moreover, a study in a mouse model for SMA has shown this compound to have an effect in this disease [12].

Therefore, we aimed to determine whether interference with the downstream signalling cascade of EphA4 may be a therapeutic strategy for motor neuron degenerative disorders. To this end, we have been studying the effect of ROCK inhibition on the motor neuron degeneration in the SOD1G93A mouse. To do so, we have treated the mice with two different ROCK inhibitors, Y-27362 and fasudil. These compounds inhibit both ROCK1 and ROCK2 isoforms, of which Y-27362 is the most specific one. However, fasudil is an FDA-approved drug, facilitating its potential translation to humans. We treated SOD1^{G93A} mice with 30 mg/kg/day Y-27362 by oral gavage starting from P100. We found a small but non-significant delay in onset and no effect on survival (Figure 3).

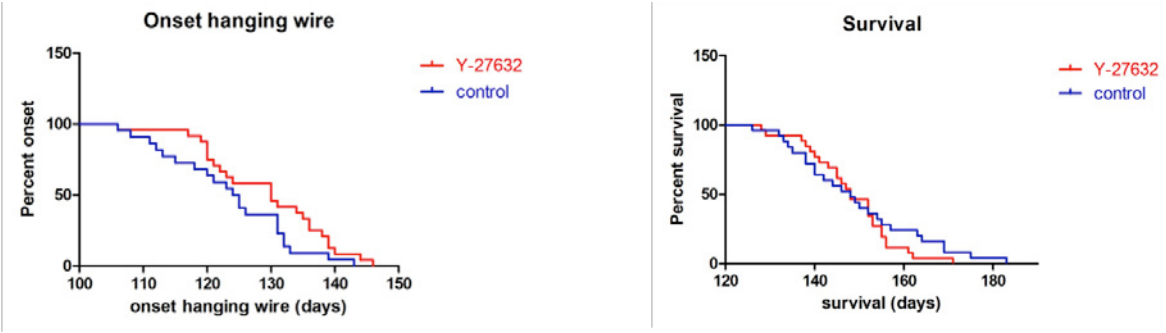

Figure 3. Treatment of SOD1^{G93A} mice with Y-27362 did not improve onset or survival. Onset analysis of control (124.5 days, n=22) and Y-27362 (130 days, n=24) treatment. Survival analysis of control (148 days, n=25) and Y-27362 (148 days, n=26) treatment.

Administering 100 mg/kg/day fasudil in drinking water has been shown to result in a therapeutic effect in mice models of various diseases. We administered 100 and 200 mg/kg/day fasudil in drinking water starting from P60. We found a significant effect of 200 mg/kg/day fasudil on onset ($p<0.05$), which was not present when using a lower doses of 100 mg/kg/day. Treatment with fasudil at any concentration had no effect onsurvival (Figure 4).

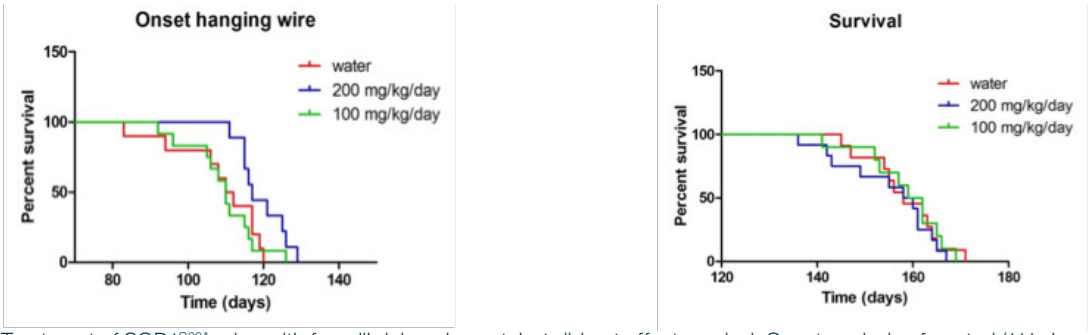

Figure 4. Treatment of SOD1^{G93A} mice with fasudil delayed onset, but did not affect survival. Onset analysis of control (111 days, n=11), 100 mg/kg/day (110 days, n=12) and 200 (117 days, n=9) mg/kg/day fasudil. Survival analysis of control (158 days, n=11), 100 (160.5 days, n=12) and 200 (159 days, n=9) treatment.

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Progress report of the research group of

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Basal Ganglia's Functions and Disorders: from Specific Genes and Signalling Pathways to Neuronal Subpopulations

Parkinson's disease (PD) and drug addiction are two major pathological conditions that rely on profound dysfunctions of the basal ganglia (BG) system. Dysfunctions of BG also occur in other conditions such as Huntington's disease, dystonia, schizophrenia or attention deficit/hyperactivity disorder (ADHD).

The BG system is composed of several interconnected subcortical nuclei including the striatum, subthalamic nucleus, globus pallidus (GP), substantia nigra (SN) and ventral tegmental area (VTA) that is involved in adaptive control of movement, motivational processes and cognitive functions. The major input to BG comes from the cerebral cortex mainly targeting the striatum, which is also under a robust regulatory influence from dopaminergic neurons of SNc and VTA and which works as the major site of integration of cortical, thalamic and midbrain afferents. BG are subdivided into a dorsal part participating in control of movement and procedural memory or skill learning and a ventral part involved in motivation and reward. 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 characterized by their outputs, either SNr or 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 BG circuitry, respectively.

The major aims of our project are to dissect out the distinct properties and to identify the precise role of striatal neuronal populations in motor control and movement disorders, procedural memory, instrumental learning and drug addiction by sub-regional optogenetic or pharmacogenetic control of specific striatal sub-populations as well as to cell-specifically and functionally characterize genes expressed in D2R-MSN and/or D1R-MSN.
The work completed in 2014 thanks to the support from QEMF is summarized below.

1. Identification of roles of D1R-MSN and D2R-MSN of striatal subregions in motor control, procedural and instrumental learning by specific optogenetic and pharmacogenetic approaches

We have previously reported the roles of D2R- and D1R-MSN in motor activity, motor learning and reward processes by using a regional and subregional cell-specific ablation models (Durieux et al., 2009, 2011, 2012). These models allowed a functional cell-type dissection of different striatal regions with a reasonable spatial resolution, but are not reversible and could lead to compensation that could preclude adequate interpretation of the data. To circumvent these issues we developed two strategies called optogenetics and pharmacogenetics using light or engineered G-protein coupled receptors, respectively, to reversibly control, in vivo or ex vivo, the activity of genetically targeted neuronal populations.

We used the light-sensitive cation channel Channelrhododopsin-2 (ChR2), opened by emission of blue light, and the Archaerhodopsin-3 (ArCh) proton pump, activated by green-yellow light, to either activate or silence neurons, respectively. Ex vivo, we tested the functional expression of optogenetic transgenes in D1R and D2R (Figure 1) neurons using whole-cell patch clamp recording technique. As shown in our previous report, in voltage-clamp mode, photostimulation of ChR2-transduced D1R- (Figure 1 A, B) and D2R-neurons with blue light caused inward current. In current-clamp experiments, neurons that had been transduced with ChR2, 5ms pulses of blue-light, caused action potentials with 100%

efficiency at 20 Hz (Figure 1 C). By contrast, we now showed that neurons transduced with ArCh (Figure 1 D, E) showed rapid outward photocurrents in voltage clamp recording (Figure 1 F). In current clamp experiments, ArCh-transduced neurons were hyperpolarized by green light, which completely blocked endogenous action potentials (Figure 1 G). These *in vitro* results show that AAV-mediated expression of ChR2 or ArCh can stimulate or inhibit action potentials with a millisecond timescale precision, respectively, in the D1R- and D2R-striatal neurons. To develop the technique in vivo in the lab, we acquired and installed specific laser systems coupled to implanted optical fibers that allow to deliver optical stimulation deep into the brain area of interest in freely moving animals. After having previously assessed the efficiency of ChR2 activation in a circling behavior as a validation assay, we are now currently testing the behavioral outcomes of light-induced neuronal activity from the different striatal subpopulations in different learning tasks.

Another alternative to cell-specific ablation has been newly developed in the lab using a pharmacogenetic approach called Designer Receptors Exclusively Activated by Designer Drugs (DREADD). This technique consists in the Cre-dependent expression of a mutated muscarinic G(iα)protein-coupled receptor (hM4Di) in striatal neurons. This approach allows a direct, non-invasive remote control of neuronal activity with high cell-specificity through G-protein coupled receptor (GPCR) signalling pathway. hM4Di receptors are exclusively activated by an inert synthetic ligand clozapine-N-oxide (CNO), which penetrates the blood-brain barrier and results in striatal neurons hyperpolarization for several hours, consistent with most drugs mechanisms of action. Indeed, one of the big advantage to use DREADD technique is that it mimics the best pharmacotherapeutic strategy to-date in human medications through GPCR signalling but it is adding a high neuron-targeted specificity, which would counteract drugs unspecific actions and side effects.

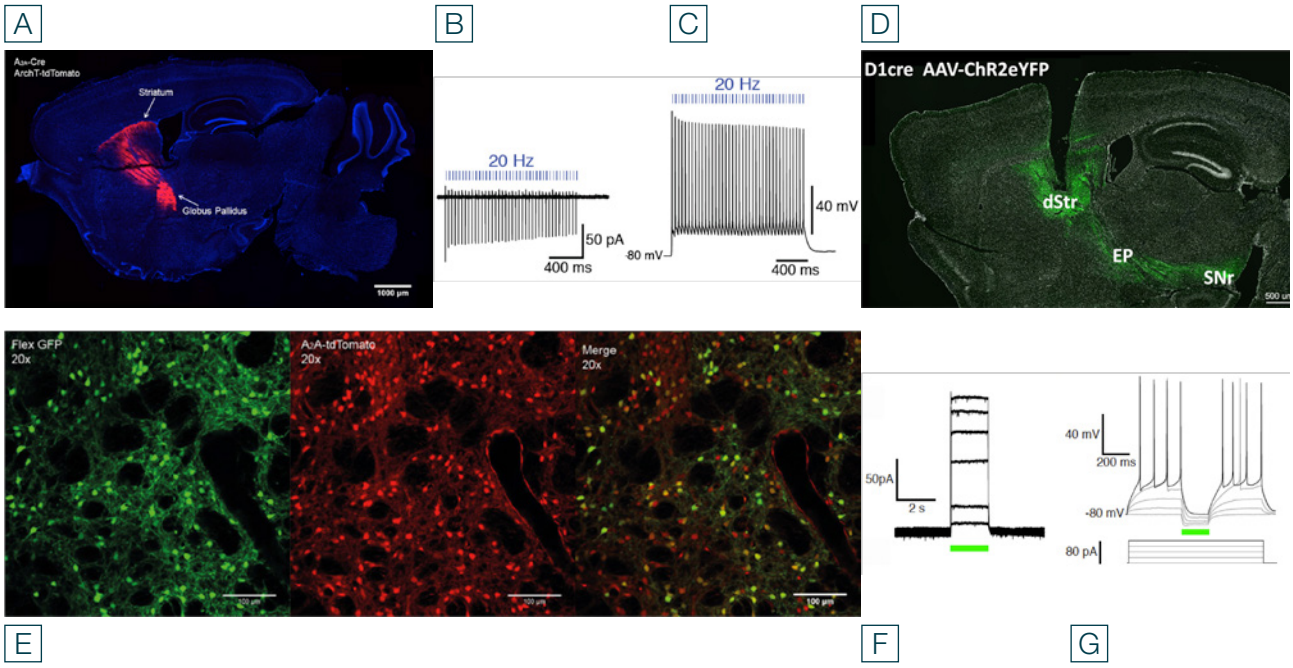

Figure 1: Specific and efficient functional expression of optogenetic transgenes in D1R- and D2R-neurons. (A) Representative photomicrographs depicting viral ChR2eYFP expression in a subpopulation of D1Rneurons. (B) Voltage clamp recording of a D1R-neuron expressing ChR2 in striatum slice showing inward currents in response to blue light pulses. (C) Blue-light pulse trains (5ms pulse) evoked action potentials trains at 20Hz in a D1R-neuron expressing ChR2. (D) Representative photomicrographs depicting viral ArChtd-Tomato expression in a subpopulation of D2R-neurons. (E) Representative photomicrographs depicting viral GFP expression (left column, green), td Tomato expression from a A2A-Cre x Rosa26 tdTomato mouse (center column, red), and merged images (right column) from an animal unilaterally injected with Flex-GFP AAV virus into the left striatum. (F) Voltage clamp recording of a D2R-neuron expressing ArCh in striatum slice showing outward currents in response to green light. (G) Action potential trains recorded under current clamp conditions from a D2R-neuron transduced with ArChtd-Tomato in striatum slice.

2. Properties of striatal fast-spiking interneurons deficient in parvalbumin and their synaptic connections to MSN and roles of FSI of striatal subregions in motor control, procedural and instrumental learning.

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

Striatal fast spiking interneurons (FSI) modulate the output of the striatum by providing a powerful feedforward inhibition on striatal principal neurons (MSN) and synchronizing their activity. Several studies have broadened our understanding of FSI, showing that they are implicated in severe disorders as Parkinsonism, dystonia, autism spectrum disorders (ASD) and Tourette syndrome. FSI are the only striatal neurons to express the Ca^{2+}-binding protein parvalbumin (PV). We have previously demonstrated, by using PV-EGFP::PV-/- mice and double patch recording as well as computer simulation, that in FSI, PV tightly regulates the calcium dynamics and is crucial for the fine-tuning of the temporal responses of the FSI network and for the orchestration of MSN populations (Bischop et al., 2012; Orduz et al., 2013).

Since the network of PV-expressing interneurons has gained particular attention in ASD but little is known on PV's putative role with respect to ASD, we pursued our study by analyzing both the behavior of PV-depleted mice in terms of ASD symptomatology, their cortico-strialal synaptic transmission and the morphology of striatal FSI (Wöhr et al., 2014). We showed that three core symptoms present in ASD patients were detected in these mice: impaired social interactions, reduced communication and repetitive and stereotyped behavior. We showed that both inhibitory and excitatory synaptic transmissions were altered. Indeed, we observed altered short-term synaptic plasticity of the excitatory cortical input to the striatal FSI. Our results suggested that short-term plasticity at this cortical neuron-FSI synapse might have a presynaptic adaptation in the cortical neuron caused by the absence of PV in the postsynaptic FSI, besides PV's more direct role at the presynaptic side in FSI and other PV^{+} interneurons. Finally after filling the recorded striatal FSI, 3D reconstruction was performed by using confocal microscopy. We showed that the number of branches was clearly increased in striatal $PV^{-/-}$ FSI and Sholl analysis revealed a higher number of dendrites in a region between 40 to 150 µm from the soma and also more branches from the 3rd to 5th order. In view of these results and knowing that changes in PV expression pattern have been reported in numerous mouse ASD models, we propose a convergent pathway in ASD, where mutations in ASD-linked genes may lead to a down-regulation of PV resulting in the ASD phenotype (Wöhr et al., 2014).

Our next questions were to identify the roles of these neurons and their PV-regulated Ca^{2+} dynamic in the cortico-striatal circuit and to evaluate in vivo the role of these neurons and their coupling by using optogenetics. For this, we started the specific development and validation of optogenetic for FSI in the striatum. We genetically targeted expression of ChR2 to PV+ cells in order to selectively activate FSIs with 470 nm light pulses. We injected PV-Cre mice with AAV conditionally expressing the fluo-tagged ChR2-tdTomato, thereby achieving specific ChR2 expression in PV+/FSIs. Those AAV contain a double-floxed inverted open reading frame encoding the ChR2-eYFP protein. Striatal neurons expressing ChR2-tdTomato are only present in Cre+ mice and they account for a sparse neuronal population. These neurons are aspiny and PV-positive; hence confirming the genetic restriction of ChR2 to FSI. Whole-cell recording was performed on tdTomato-expressing neurons in cortico-striatal slices from these mice. We characterized the activation of these neurons by delivering continuous or pulsed illumination (Figure 2 A,B). Fluorescent neurons which depolarized upon optical stimulation were shown to be FSI (n = 10 of 10) as determined by their electrophysiological properties such as non-accommodating discharge pattern, high discharge rate, narrow action potentials, and fast and deep afterhyperpolarization. FSI depolarised strongly, leading in all cases (n = 10) to action potential discharges. Light pulses of 2 ms duration were usually sufficient to induce these action potentials and this was observed for a broad

range of frequencies. Inhibitory post-synaptic current (IPSC) were recorded in MSN (Figure 2 C,D). FSI contacting neighboring MSN provided inhibition (n = 6 of 6; Figure 2 C,D) since light presentation paused firing evoked by current steps (Figure 2 D). Light-induced synaptic responses are $GABA_A$-R dependent. We have therefore validated the model *in vitro* and the next steps will be 1) to analyze the network properties of these FSI *in vitro* in terms of their control of MSNs - the striatal principal neurons – activity and 2) to implement the strategy to correlate these data to the involvement of these neurons on striatal-dependent behavior. Bilateral excitation of the striatum will allow us to assess the role of the FSI microcircuits on the behaviour of awake animals.

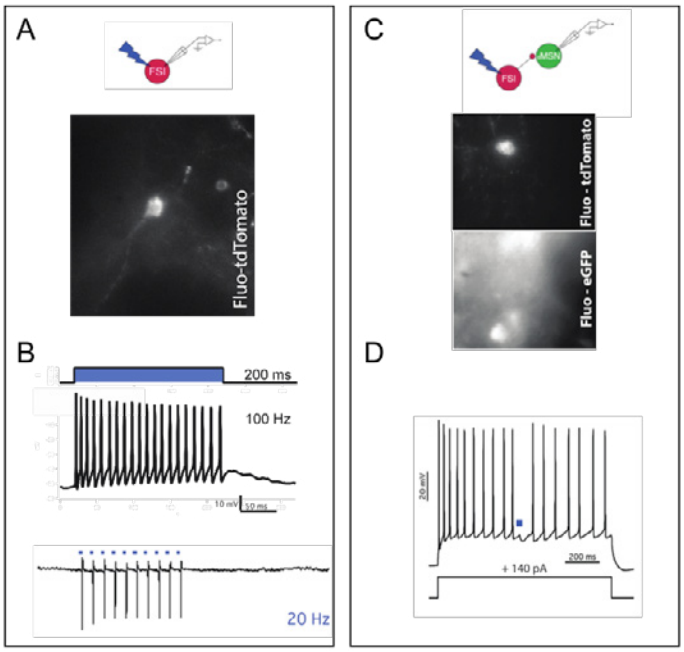

Figure 2: (A) Optogenetic control of striatal fast spiking interneurons (FSI) in acute brain slices from Pvalb-Cre mice injected with ChR2-tdTomato AAV. Recorded FSI is visible in fluorescence. (B) Train of action potentials evoked by a continuous 200ms light pulse, recorded using the whole configuration of the patch-clamp technique, and train of action potentials evoked by a 20Hz train of 2ms light pulses, recorded in the cell attached mode. (C) Striatal medium spiny neuron (MSN) recorded in slices from a Pvalb-Cre x ChR2-tdTomato x D1-GFP mouse. The ChR2 expressing is FSI is tagged with tdTomato and the recorded MSN is labelled with GFP. (D). Optogenetic activation of a FSI by a 50ms light pulse (blue line) induces the inhibition of action potential firing in a targeted MSN. (L. Lambot, *unpublished results*)

3. Specific inactivation of NR1 in D2R-striatopallidal neurons

Neuroadaptation and more specifically synaptic plasticity involve several important neurotransmitter receptors and intracellular signalling cascades. Among the involved receptors, the Ca^{2+} permeable glutamate NMDA receptor is a central and initial player. This has been firmly demonstrated at different excitatory synapses such as in the hippocampus (Tsien et al., 1996). The NMDA receptor seems to have key influence in the mechanisms of reward and addiction as well as in motor skill learning. Synaptic plasticity events could be very different in striatopallidal and striatonigral neuronal subpopulations and must have diverse functional and behavioral consequences. In particular, the role of the NMDA-R-initiated signaling pathways in long-term synaptic plasticity in the striatopallidal neurons and their involvement in normal motor learning and striatal neuroadaptation to dopamine depletion, or drug addiction were mostly undetermined. We have generated A_{2A}R-Cre::Grin1$^{f/f}$ mice to specifically inactivate the essential NR1 subunit of the NMDA receptor in striatopallidal neurons.

These mice have been used at 2 months of age for testing their behaviour in relationship to locomotor activity, motor learning and attentional behaviour. The results showed that these mice exhibit motor dysfunctions with spontaneous hyperlocomotion. Indeed, in an *open field* test, A_{2A}R-Cre$^{+/-}$ Grin1$^{f/f}$ mice exhibit a higher locomotor activity as compared to their control A_{2A}R-Cre$^{-/-}$ Grin1$^{f/f}$ littermates. Moreover, when tested on three successive days, unlike control mice, which have a significantly decremental exploratory activity across sessions, the activity of A_{2A}R-Cre$^{+/-}$ Grin1$^{f/f}$ mice remains stable. The motor skill learning was then compared by the rotarod and runway tests. In the accelerant rotarod test, mice

learn a new sequence of movements to maintain balance on a rotating rod with constant acceleration. When testing on the runway, mice must run along an elevated line dotted with small obstacles intended to impede their progress. The number of shifts of the hind paw on the visible side of the experimenter is recorded. The analysis of mice showed no significant difference between $A_{2A}R\text{-}Cre^{+/-}$ $Grin1^{f/f}$ mice and their controls in motor learning performance during the rotarod and runway tests. However during the accelerating rotarod task, $A_{2A}R\text{-}Cre^{+/-}$ $Grin1^{f/f}$ mice deficient in NMDA receptor were turning on themselves in the horizontal plane simultaneously with executing their task. Quantification of this behaviour showed a high significance as compared to control mice. Interestingly, these locomotor deficits are similar to those observed following the selective ablation of D_2R striatopallidal MSN (Durieux et al., 2009, 2012), suggesting that NMDA receptor is required for both learning and spontaneous motor behaviour. In view of these putative attentional deficits, a novel object recognition test and instrumental learning task in boxes were performed. The novel object recognition test showed an increase in exploration of both novel and old objects but failure to discriminate between these two objects in the $A_{2A}R\text{-}Cre^{+/-}$ $Grin1^{f/f}$ mice. Again this attentional deficit is similar to what was observed following the selective ablation of D_2R striatopallidal MSN (Durieux et al., 2011) and suggests that NMDA receptor in this neuron population is required for selective attention processing. The instrumental learning (or operant conditioning) was performed in a behavioral paradigm of positive reinforcement where the animals must learn to associate an action (placing the nose in a hole, Nose Pokes NP) to its specific result (distribution of granules sweet) and then gradually increase their rate of NP until a stable plateau value. The average NP obtained by the $A_{2A}R\text{-}Cre^{+/-}$ $Grin1^{f/f}$ and control mice during appetitive operant conditioning did not differ significantly. Then, a protocol of devaluation was tested to highlight the transition from appetitive character to a goal-directed behavioral habituation. This resulted in a decrease of the instrumental response in the two groups but there was a significantly lower devaluation in $A_{2A}R\text{-}Cre^{+/-}$ $Grin1^{f/f}$ than in control mice. In addition, the number of visits to the feeder during the conditioning period was significantly more important for $A_{2A}R\text{-}Cre^{+/-}$ $Grin1^{f/f}$ than in control mice supporting again their increased exploratory behavior.

Locomotor sensitization to psychostimulants is widely used in the evaluation of drug reinforcement and drug addiction mechanisms. Drug sensitization is best characterized for the locomotion activation induced by psychostimulants as cocaïne or amphetamine in rodents and consists in an increase in locomotor activity in response to a fixed dose of drug given intermittently (for example daily). We evaluated involvement of NMDA receptor in striatopallidal neurons in these motor responses to psychostimulant by quantifying the presence and intensity of amphetamine-induced locomotor sensitization in $A_{2A}R\text{-}Cre^{+/-}$ $Grin1^{f/f}$ as compared to control mice. Both groups exhibited a similar acute increase in locomotor activity following the first amphetamine injection and demonstrated a locomotor sensitization following repeated amphetamine administration. However, the amplitude of this locomotor sensitization was significantly lower in $A_{2A}R\text{-}Cre^{+/-}$ $Grin1^{f/f}$ than in control mice.

Therefore altogether, these results showed that NMDA receptors in striatopallidal neurons are required for the regulation of motor and attentional behaviour (spontaneous locomotion, reactivity to novelty) and for motivational and reward processes (instrumental learning and conditioning to psychostimulants).

To establish correlation between these behavioural alterations and putative neuroadaptative changes in the striatal microcircuit we have performed an electrophysiological characterization of these neurons. In order to allow the identification of neurons deficient in NR1 in brain slices for patch clamp recordings, $A_{2A}R\text{-}Cre^{+/-}$ $Grin1^{f/f}$ and $A_{2A}R\text{-}Cre^{-/-}$ $Grin1^{f/f}$ mice were further crossed with reporter mice (LoxP-Stop-LoxP-YFP) leading to the expression of *Yellow Fluorescent Protein* (YFP) in recombined neurons.

NMDA currents have been identified in whole-cell configuration in corticostriatal brain slices. A stimulation electrode is placed in the white matter (corpus callosum) separating the striatum from

the cortex and permits stimulation of corticostriatal axons and recording of EPSC in targeted striatal neurons. A protocol allowing the extraction NMDA-R of the EPSC has been developed based on the differential kinetics of the NMDA-R- and AMPA-R-mediated components. Recordings in both groups of mice showed that there was a total absence of the NMDA receptor-mediated component of the EPSC in most YFP-positive neurons in A_{2A}R-Cre$^{+/-}$ Grin1$^{f/f}$ mice (Fig. 3) with a strong and significant decrease of the averaged NMDA-R component in A_{2A}R-Cre$^{+/-}$ Grin1$^{f/f}$ as compared to control mice.

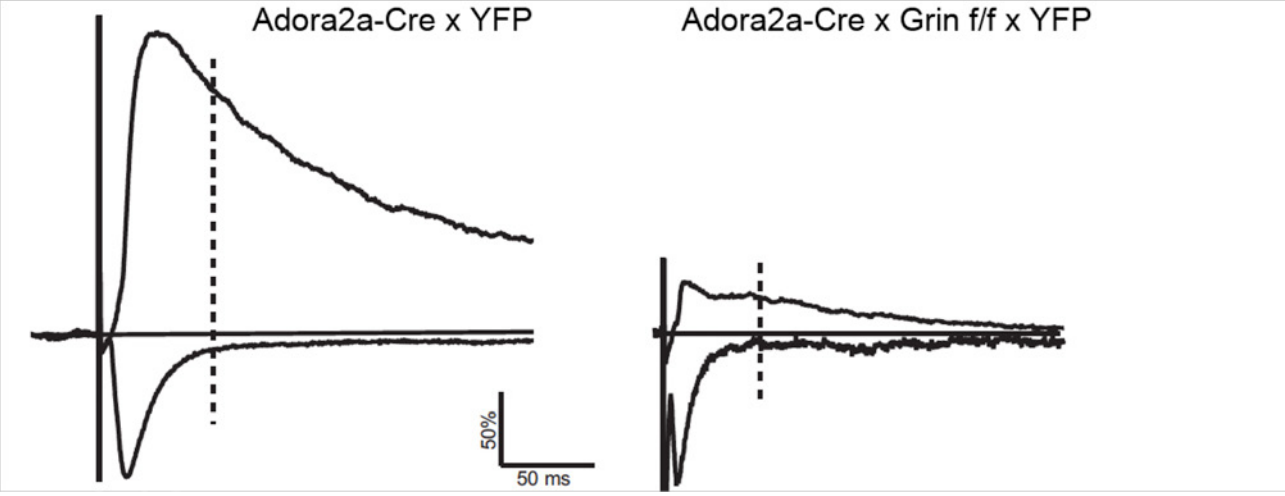

Figure 3: Absence of the NMDA-component of evoked EPSCs in D2R-MSN in slice from Adora2a-Cre x Grin f/f x YFP as compared to a control D2R-MSN. In each cell, EPSCs were evoked at -70 mV and +40 mV.

Since the chronic absence of excitatory drive may alter excitability of neurons, we evaluated the intrinsic excitability of these NR1-deficient striatopallidal neurons. These recordings showed that the resting membrane potential is increased, the rheobase is decreased and the excitability is increased as demonstrated in the current/frequency plot as compared to the control cells (Figure 4). Moreover, these neurons are also more prone to accommodation. Altogether, these results showed that the NR1-deficient striatopallidal neurons are hyperexcitable, probably through a mechanism of homeostatic plasticity. This part of the project is now pursued by the analysis of the neuronal morphology (spines density, …) by 3D reconstruction of the recorded neurons.

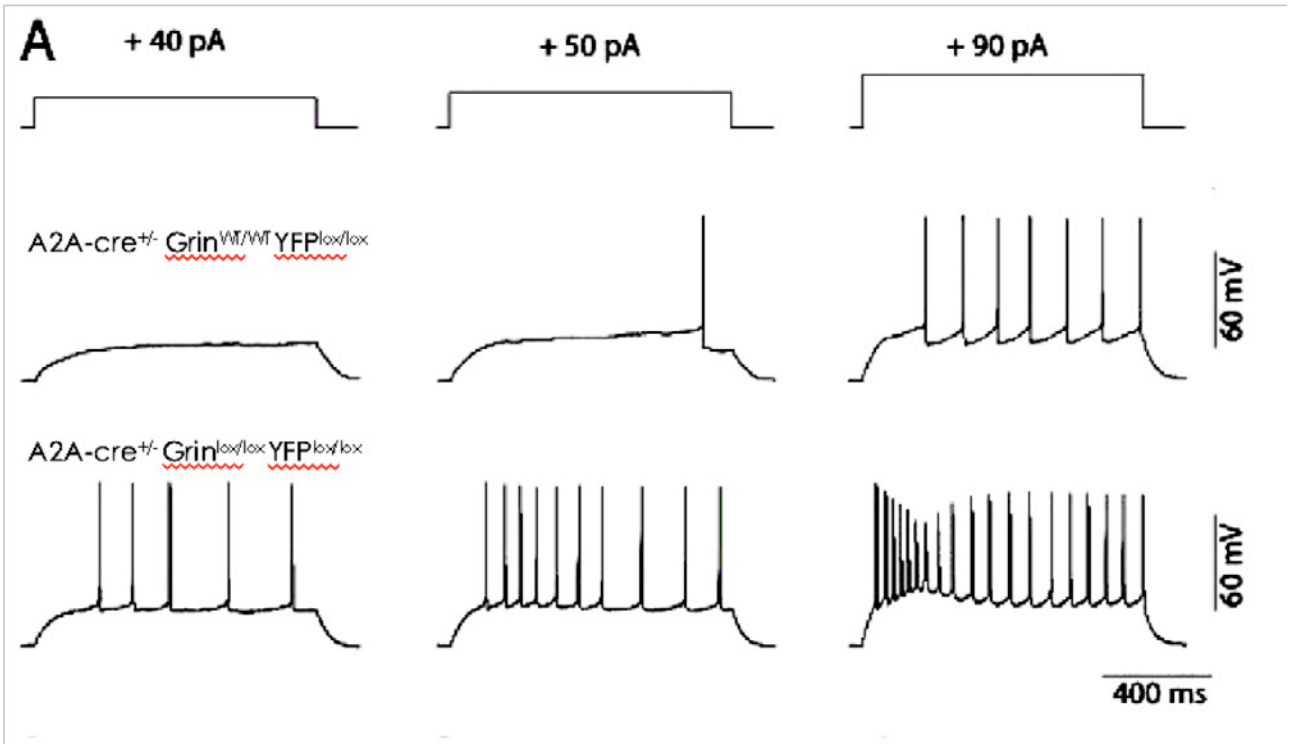

Figure 4: excitability of control (top) and NR1-deficient (bottom) striatopallidal neurons in response to injection of current of increased amplitude.

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 previously set up protocols to purify MSN subpopulations by FACS-sorting of samples prepared from GFP-striatopallidal (A_{2A}R-Cre::Z/EG) mice retrogradely labelled for striatonigral MSN. We succeed in isolating green (striatopallidal) and red (striatonigral) neurons from individual mice with a very good rate of enrichment and have established gene profiles of these neurons by micro-arrays showing more than 200 striatopallidal neuron specific genes and more than 400 striatonigral neuron specific genes (> 2 fold differential expression) (Ena et al., 2013). This differential gene expression has been validated by using different techniques for a series of genes. We have previously functionally characterized one of these genes, the ecto-nucleotidase NTe5 selectively expressed in striatopallidal neurons (Ena et al., 2013). We have now selected a series of additional genes that are selectively expressed in one MSN subpopulation, that we are characterizing in terms of their functional involvement in the basal ganglia system and its pathologies by using conditional gene inactivation.

One of these genes encodes the β receptor isoform of platelet-derived growth factor, PDGFRβ. PDGF is a growth factor whose action is mediated by two receptors with tyrosine kinase activity. In neurons, PDGFRβ is the predominant receptor level, and, when activated by PDGF-BB, is responsible for the activation of various intracellular signaling pathways. PDGFRβ can also be activated in the absence of its ligand by a transactivation mechanism, resulting from the activation of G protein-coupled receptor such as 5HT1a or D2 receptors. The previous results obtained by microarray have been validated by qPCR and also now by immunofluorescence and showed a preferential expression of PDGFRβ in striatopallidal MSN. Given the specific expression of D2R in these neurons, the interaction between PDGFRβ and D2-R could therefore also take place.

In order to characterize the functions of this receptor in these neurons, we started protocols of specific invalidation of PDGFRβ in striatopallidal neurons. We are crossing mice bearing a floxed allele of PDGFRβ gene (PDGFRβ$^{f/f}$ mice) and A_{2A}R-Cre mice (Durieux et al., 2009, 2012) or D2R-Cre mice. In parallel, as an alternative, stereotaxic injections of AAV expressing Cre recombinase in the PDGFRβ$^{f/f}$ mice striatum were also performed. In this latter model, immunofluorescence showed a reduction in the striatal PDGFRβ expression, confirming its validity. Behavioral analysis of the resulting mice devoted in PDGFRβ in striatopallidal neurons will be carried out to investigate its role locomotor activity, motor learning and procedural learning as well as in motivational processes and drug addiction by using devices and tests available (Durieux et al., 2009, 2012; Ena et al., 2013).

In order to characterize cell signaling pathways initiated by PDGFRβ in striatopallidal neurons, we performed primary cultures of wild mice striatal neurons. The PDGFRβ activation state (as detected by the phosphorylation T1021 residue) and the activation of a downstream signaling MAP kinase pathway (as detected by p-ERK 1/2) is examined by Western blotting. We have shown that phosphorylation of ERK1/2 induced by PDGF-BB in striatal neurons is dependent on PDGFRβ because this effect is suppressed in the presence of TAG1296, an inhibitor of PDGFRβ. The effect of the activation of D2R on the activation of PDGFRβ and downstream signaling pathways has also been studied by using quinpirole, a specific D2R agonist. We are currently trying to identify the cellular factors involved in the signalling pathway.

It has been showed in the hippocampus that PDGFRβ activation results in inhibition of NMDA receptor-dependent currents. In view of the importance of these receptors in striatopallidal neurons functions (see above), we therefore started to examine the regulatory role of PDGFRβ in striatopallidal neurons by using the patch clamp technique. Preliminary results showed that application of PDGF-BB on striatal slices induced a decrease in NMDA current in striatopallidal neurons in a similar proportion to that observed in the hippocampus. These results will be extended and validated by appropriate controls and the effect of PDGF-BB on cortico-striatal synaptic plasticity involving NMDA receptors will be also conducted.

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

- Phosphodiesterase 10A (PDE10A) is located mainly to the striatal medium spiny neurons (MSN) and hydrolyses cAMP and cGMP, key determinants of MSN signaling. We showed that genetic depletion of PDE10A critically mediates attribution of salience to reward-predicting cues, evident in impaired performance in PDE10A knockout mice in an instrumentally conditioned reinforcement task. We furthermore reported modest impairment of latent inhibition in PDE10A knockout mice, and unaltered prepulse inhibition. We suggested that the lack of effect on PPI is due to the pre-attentional nature of this task. Finally, we performed whole-cell patch clamp recordings and showed changes in intrinsic membrane excitability. A decrease in spontaneous firing in striatal medium spiny neurons was found. These data show that PDE10A plays a pivotal role in striatal signaling and striatum-mediated salience attribution (Piccart et al., 2014).

- A2AR-D2R heteromers are key modulators of striatal neuronal function. It has been suggested that the psychostimulant effects of caffeine depend on its ability to block an allosteric modulation within the A2AR-D2R heteromer, by which adenosine decreases the affinity and intrinsic efficacy of dopamine at the D2R. We described novel unsuspected allosteric mechanisms within the heteromer, by which not only A2AR agonists but also A2AR antagonists decrease the affinity and intrinsic efficacy of D2R agonists and the affinity of D2R antagonists. Strikingly, these allosteric modulations disappear upon

agonist and antagonist co-administration. This can be explained by a model that considers A2AR-D2R heteromers as heterotetramers, constituted by A2AR and D2R homodimers, demonstrated by experiments with bioluminescence resonance energy transfer and bimolecular fluorescence and bioluminescence complementation. As predicted by the model, high concentrations of A2AR antagonists behaved as A2AR agonists and decreased D2R function in the brain (Bonaventura et al., submitted).

- Glucocorticoid receptor (GR) function is impaired in multiple brain disorders, particularly in aging and Alzheimer's disease (AD). We showed that human aged and AD brains exhibit a striking adenosine A2A receptor (A2AR) overexpression which is associated to GR-down-regulation, but the causal link remained unknown. In this work, we demonstrated that a forebrain neuronal-selective increase in A2AR is sufficient to induce HPA-axis dysfunction and aging-like hippocampal deficits, namely memory task impairments. Furthermore synaptic plasticity deficits triggered by a GR agonist were exacerbated by A2AR over-activation, while GR transcription and nuclear translocation were prevented under A2AR blockade. Finally, A2AR antagonist therapy increased histone H3 acetylation associated with the GR gene-*Nr3c1*. Taken together, these findings demonstrate that GR mediated responses are directly modulated by A2AR providing an alternative to current GR targeted clinical interventions (Batalha et al., submitted).

- Calcium binding proteins regulate intraneuronal Ca^{2+} homeostasis and hence, supposedly, if present in presynaptic terminals, neurotransmitter release. As expected, absence of the fast Ca^{2+} buffer calbindin (CB) leads to increase in presynaptic action potential-evoked $[Ca^{2+}]_i$ transients at the Purkinje cell (PC) recurrent synapses whereas, unexpectedly, IPSC mean amplitudes remained unaltered in connected CB-/- PC. To explain these paradoxical observations, we hypothesized that morphological compensation/adaptation mechanisms might be induced in CB-/- PC axon collaterals including boutons. By using biocytin-filled PC 3D-reconstructions and electron microscopy, we showed larger bouton volume, increased active zone length and a higher number of docked vesicles, in combination with an increase in synaptic cleft width. We propose that these morphological changes likely modify the GABA release properties at this synapse in CB-/- mice and suggested that these changes act as adaptation/homeostatic mechanisms to likely preserve characteristics of synaptic transmission in the absence of the fast Ca^{2+} buffer CB (Orduz et al., 2014).

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Progress report of the research group of

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Shaping the nervous system: Role of the planar cell polarity genes

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 have demonstrated a key role for Celsrs in the planar cell polarity (PCP) pathway. PCP is complementary to the intrinsic polarization of single cells and refers to the global coordination of cell behaviour in the plane of a tissue. Most evident in cell sheets and confined to studies in Drosophila for years, PCP has emerged during the last decade as an important pathway in vertebrates, where it regulates various developmental processes and is associated with multiple disorders.

When we started studying the mammalian Celsr genes in early 2000s, two members (*Celsr1* and *Celsr2*) were listed in databases, but little 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 last year, we have progressed on the following points:

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.

During the last year, we pursued our work in the central nervous system, we analyzed genetically the contribution of Celsr2, Celsr3, Fzd3, and Vangl1, Vangl2 to axonal development in the forebrain, by focusing on the anterior commissure (AC), the corticospinal tract (CST), and the internal capsule (IC). The AC contains commissural axons from the anterior olfactory nuclei and from the temporal cortex, which cross the midline at embryonic day 13.5 (E13.5) to E14.5. The IC contains three main axonal components. Thalamocortical axons (TCA) emerge from the thalamus, formerly called “dorsal” thalamus at E12.5. They run through the prethalamus (former “ventral” thalamus), turn and cross the diencephalon–telencephalon junction, progress through a corridor in the ventral telencephalon, and cross the pallial–subpallial boundary to reach the cortical anlage from E14.5. Corticothalamic axons (CTA) emerge initially from neurons in the subplate and future cortical layer 6, around E13.5. They cross the pallial–subpallial boundary and progress in the ventral telencephalon, in opposite direction to TCA. They cross the diencephalon–telencephalon junction and begin to enter the thalamus at E14.5. Subcerebral projections such as the CST begin to leave the cortical plate at E14.5–E15.5 to enter the IC, and diverge from CTA to form the cerebral peduncle, en route to their subcortical targets such as the spinal cord. Previous work showed that Celsr3 is required for the development of the AC, IC, and CST, and Cre-

mediated regional inactivation indicated that axon navigation requires Celsr3 expression in neurons of origin and/or in guidepost cells along the pathway. The Celsr3 mutant axonal phenotype is quite similar to that in Fzd3 mutant mice, hinting that a PCP-like mechanism may regulate axon progression via interactions between growth cones and guidepost cells. To understand further the role of membrane-associated core PCP proteins in axon guidance, we used a panel of mutant mice and show genetically that Celsr2 and 3 regulate the formation of forebrain axon bundles in a redundant manner, and are required in the same cell populations as Fzd3. Unlike epithelial PCP, however, the action of Celsr2-3 and Fzd3 on forebrain axonal fascicles is Vangl1,2 independent. Inactivation of Celsr2-3 or Fzd3 in thalamus generates no evident phenotype, showing that the derailed TCA phenotype in constitutive mutants is non-cell autonomous. Furthermore, we showed that the joint inactivation of Celsr2-3 or Fzd3 in thalamus and cortex perturbs the development of cortical sensory maps. This work was published in *Proc Natl Acad Sci U S A* (July 2014). The pdf file of the paper is attached (appendix 1).

To explore the role of Celsr3 in the peripheral nervous system, we studied the impact 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 surficial versus deep peroneal nerve 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 elicit the attractive signal of GDNF. By contrast, Celsr3 and Fzd3 mutant motor neurons, contrary to wild-type 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 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 was published in *Nature Neuroscience* (September 2014). The pdf file is attached (appendix 2).

2. Celsr1-3 in multiciliated 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. We showed previously 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. This work was published in *Proc Natl Acad Sci USA* (July, 2014). The pdf file is attached (appendix 3).

Finally, with two Japanese groups, we analyzed the roles of Celsr1 in the development of the mouse oviduct ciliated cells. We found that as in the ependyma, Celsr1 was required for the orchestration of the direction of ciliary movements, the proper epithelial cellular shapes and arrangements, and the orderly patterned alignment of epithelial folds in the longitudinal direction in the oviduct. This work has just been accepted for publication in *Development*.

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   IF: 6.7
2. Celsr3 is required in motor neurons to steer their axons in the hindlimb. Chai G, Zhou L, Manto M, Helmbacher F, Clotman F, Goffinet AM, Tissir F.
   Nature Neuroscience 2014; 17: 1171-1179.
   IF: 15.25
3. A Dual role for planar cell polarity genes in ciliated cells. Boutin C, Labedan P, Dimidschstein J, Richard F, Cremer H, André P, Yang Y, Mireille Montcouquiol M, Goffinet AM, Tissir F. A Dual role for planar cell polarity genes in ciliated cells.
   Proc Natl Acad Sci 2014; 111: E3129-3138
   IF: 10.58
4. Genetic evidence that Celsr3 and Celsr2, together with Fzd3, regulate forebrain wiring in a Vangl-independent manner. Qu Y, Huang Y, Feng J, Alvarez-Bolado G, Grove EA, Yang Y, Tissir F, Zhou L, Goffinet AM.
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Endoplasmic Reticulum stress in autoimmune central nervous system inflammation and demyelination

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS). MS is prevalent in Caucasians, where it affects about 0.05%-0.15% of the population. The cause of degeneration in MS remains largely enigmatic, but is generally considered to be the result of an autoimmune inflammatory reaction leading to demyelination, oligodendrocyte loss and axonal damage in the CNS. The disease is characterized by activated auto-reactive myelin-specific lymphocytes that home to the CNS where they initiate a vicious cycle of inflammation and tissue damage. The major targets in MS pathology are oligodendrocytes, the myelin-producing cells of the CNS, and neurons, and their loss is directly associated with clinical manifestations of the disease, including speech disturbances, sensation deficits and paralysis. Much knowledge concerning MS pathogenesis has resulted from studies on its animal model Experimental Autoimmune Encephalomyelitis (EAE).

Endoplasmic reticulum (ER) stress is likely to be a major pathway in the pathogenesis of MS. ER stress occurs upon the accumulation of unfolded or misfolded proteins in the ER initiating the **unfolded protein response (UPR).** The UPR has to deal with ER stress by increasing the folding capacity of the ER by reducing protein synthesis and promoting protein degradation though ER-associated degradation. Three different signalling cascades can be activated (Figure 1): the inositol-requiring transmembrane kinase/endonuclease 1 (IRE1) pathway, the pancreatic ER kinase (PERK) pathway and the activating transcription factor 6 (ATF6) pathway. Although all three branches are usually activated by any given ER stress event, the timing of activation can differ.

ER stress is part of normal cellular physiology, but can, however, become problematic in conditions of chronic, non-resolved stress, giving rise to inflammation and/or apoptosis. Recent observations suggest that the signalling pathways in the UPR and those controlling inflammation are interconnected and can activate each other through various mechanisms including the activation of NF-κB and MAP kinases (Zhang and Kaufman, 2008), suggesting that ER stress contributes to the pathology of many inflammatory diseases, including MS. Indeed, evidence is emerging that the UPR is involved in the disease pathogenesis of MS and EAE. Oligodendrocytes continuously produce large amounts of myelin to perform their function, making them prone to protein misfolding and ER stress. Expression of ER stress markers has been found to be upregulated in macrophages, microglia, astrocytes, and oligodendrocytes within demyelinated white matter lesions from MS patients. Moreover, elevated levels of phosphorylated-eIF2α, typical for PERK-dependent UPR signalling, have been observed in oligodendrocytes and infiltrating T-cells in the CNS during the course of EAE (Lin and Popko, 2009). Notably, IFN-γ exerts protective activities through the activation of the PERK-eIF2α pathway in oligodendrocytes (Lin et al., 2007). On the other hand, IFN-γ has suppressive activity on oligodendrocyte regeneration, inhibiting remyelination in MS and EAE demyelinated lesions (Lin et al., 2006). These data suggest that ER stress induction in fully myelinated mature oligodendrocytes promotes cell survival, but in actively (re)myelinating oligodendrocytes leads to cell death (Lin and Popko, 2009). Together, these observations clearly indicate the involvement of ER stress in MS and EAE pathology, suggesting that manipulation of the UPR may be beneficial in order to prevent disease. Since inflammation and ER stress may induce autophagy responses as a compensatory mechanism, also autophagy may be involved in MS (Adolph et al., 2013). **However, a better comprehension of the role and molecular mechanisms of UPR signaling and autophagy in MS/EAE is necessary.**

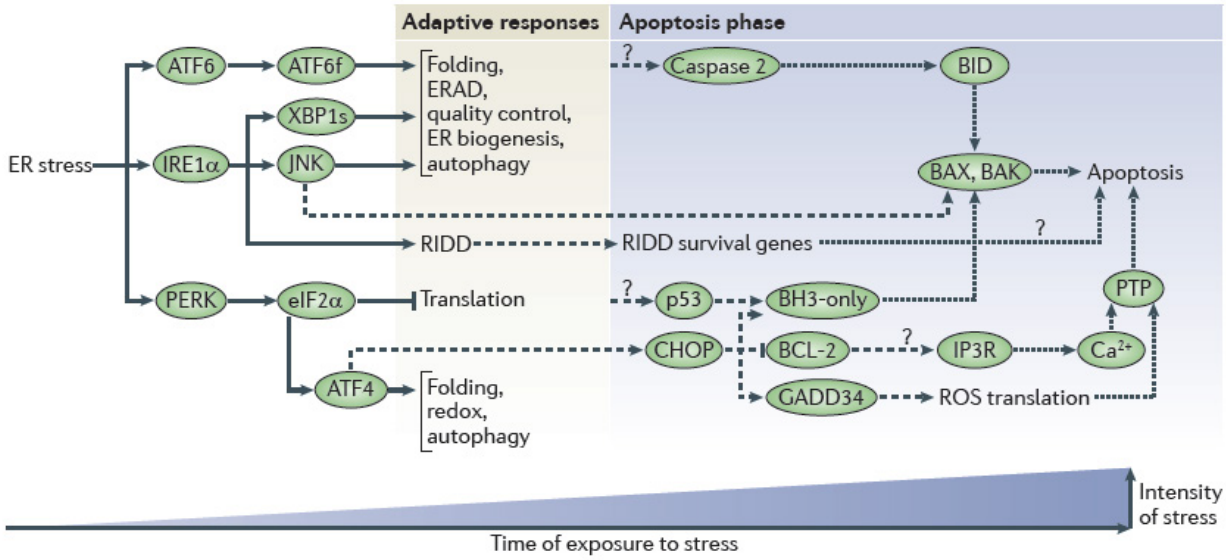

Figure 1. : ER stress cell fate decisions (Hetz, 2012). Different UPR-induced responses are observed over time in cells undergoing ER stress. Chronic ER stress induces inflammation and eventually apoptosis (through different poorly characterized mechanisms) in order to eliminate irreversibly damaged cells.

With this project we aim to better understand the contribution of autophagy and UPR signaling to the inflammatory processes associated with the development and progression of MS. The basic approach is to genetically manipulate genes coding for proteins essential for autophagy and UPR signaling in mice in specific neuronal populations, and to determine the effects of such mutation in neuronal development and MS pathogenesis. These studies will allow us to specify the role of UPR and autophagy signalling locally in the CNS, both in target cells (such as in neurons and oligodendrocytes) and in effector cells (such as in astrocytes and microglia) in the inflammatory and neurodegenerative processes happening during MS/EAE. Indeed, differences in UPR responses and sensitivity to ER stress may be expected depending on the cell type and the function they exert. It is thus critically important to determine how these various cell types at various stages of activation and disease development react to chronic inflammatory stress in order to better understanding the pathogenesis of MS (and other demyelinating diseases) which may have implications for the rational design of new therapeutics for the treatment of these pathologies.

1. Results 1st project year

1.1. Mouse conditional gene targeting

Conditional 'floxed' mice targeting NF-κB signaling (IKK2^{FL}), autophagy (Atg16l1^{FL}) and the three individual UPR signaling pathways (CHOP, XBP1^{FL}, IRE1α^{FL}, ATF6α^{FL}, PERK^{FL}) have been obtained or are in the process of being generated. Also the different Cre transgenic lines needed for CNS targeting have been introduced in the research group: Nestin-Cre transgenic mice for pan-CNS targeting, Thy1.2-Cre mice for neuron-specific targeting, MOGi-Cre mice for oligodendrocyte targeting, GFAP-Cre mice for astrocyte targeting, and Cx3Cr1-Ert2Cre mice for microglia targeting.

To study the CNS-specific role of ER stress and autophagy in the immunopathology of MS, we make use of the experimental MS model EAE, which can be induced by immunization of mice with myelin oligodendrocyte glycoprotein (MOG) or other encephalogenic agents. Next to EAE, brain-specific demyelination can also be induced by putting mice on a diet containing the neurotoxicant cuprizone. Advantage of this approach is that demyelination can afterwards be reversed by administration of normal food, allowing the study of brain remyelination and the involvement of ER stress signalling and

autophagy in this. Finally, protocols for *in vitro* studies on primary cells derived from the different tissue-specific knockout mice have been established, allowing biochemical studies complementary to the *in vivo* approaches.

1.2. CHOP-dependent neuronal apoptosis in EAE

ER stress has several downstream effects, of which inflammation and apoptosis are the two major outcomes. ER stress can activate CHOP, a protein responsible for ER stress-mediated apoptosis (Oyadomari and Mori, 2004). To determine whether CHOP plays a role in EAE pathogenesis, we induced EAE in wild-type ($CHOP^{+/+}$), heterozygous ($CHOP^{-/+}$) and CHOP deficient ($CHOP^{-/-}$) littermate mice.

Upon EAE induction, $CHOP^{-/-}$ mice did not show protection nor exacerbation of symptoms when compared to $CHOP^{-/+}$ and $CHOP^{+/+}$ mice (Figure 2 and table 1). Moreover, quantification of degree of demyelination through histology did not show any difference between the three groups of mice. Furthermore, inflammatory gene expression nor peripheral T cell activation did show any significant difference between the three genotypes (data not shown). From these observations, we can conclude that CHOP, and CHOP-dependent UPR signalling, does not crucially contribute to EAE onset and disease progression Since no difference in degree of demyelination could be observed in CHOP deficient conditions compared to controls, oligodendrocyte survival is not influenced by the absence of CHOP in conditions of EAE.

ER stress and CHOP expression can trigger apoptosis under physiological and pathophysiological conditions (Tabas and Ron, 2011). Indeed, using MEFs isolated from control and CHOP deficient mice, we could confirm the importance of CHOP in tunicamycin-induced cell death, since CHOP deficient MEFs are significantly protected from death induced by tunicamycin, a stimulus known to induce ER stress (data not shown). Although CHOP may possibly also contribute to death of oligodendrocytes in EAE, its effect may not be detectable in conditions of extensive inflammation where inflammatory cytokines such as TNF and Fas ligand may induce apoptosis independent of CHOP (Mc Guire et al., 2010).

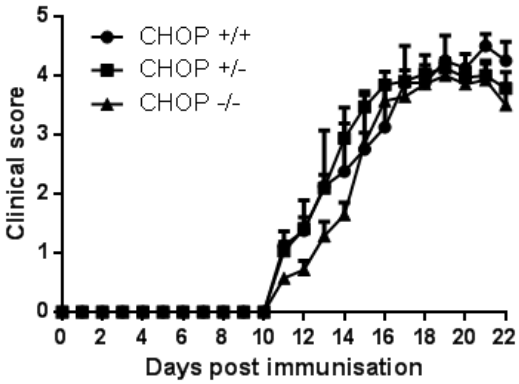

Figure 2. Clinical disease scores of $CHOP^{-/-}$ (n=7), $CHOP^{+/-}$(n=16) and $CHOP^{+/+}$ (n=4) mice after immunization with MOG peptide. Results are from three independent experiments and presented as mean values ± s.e.m.

Table 1. Clinical features of MOG_{35-55} induced EAE in CHOP mice

Genotype	Incidence	Mean day of disease onset	Maximum clinical score
$CHOP^{+/+}$	4 of 5 (80%)	14 ± 1.22	4.5 ± 0.2
$CHOP^{+/-}$	16 of 17 (94%)	13.12 ± 0.41	4.41 ± 0.13
$CHOP^{-/-}$	8 of 8 (100%)	14.38 ± 0.32	4.37 ± 0.12

Results are presented as mean values ± s.e.m.; combined data from three independent experiments.

1.3. XBP1-dependent UPR signaling in EAE

IRE1α is the most conserved transducer of UPR which acts through unconventional splicing of XBP1 mRNA. To investigate the importance of XBP1-dependent signalling in CNS inflammation, we generated CNS-specific XBP1 deficient mice and subjected them to EAE. $XBP1^{CNS\text{-}KO}$ mice, however, developed EAE pathology to the same extent as control littermates (Figure 3A). Also, on spinal cord histology nor on spinal cord gene expression, no significant differences could be observed (data not shown).

Since oligodendrocytes are particularly vulnerable to ER stress, we next investigated the response of oligodendrocyte-specific XBP1 knockout mice to EAE. However, although $XBP1^{ODC\text{-}KO}$ mice are behaving slightly better in EAE, especially at later time points, no significant differences with control mice could be observed (Figure 3B). In conclusion and opposite to what was expected, XBP1-dependent UPR signalling does not seem to be involved in autoimmune-mediated inflammation and demyelination.

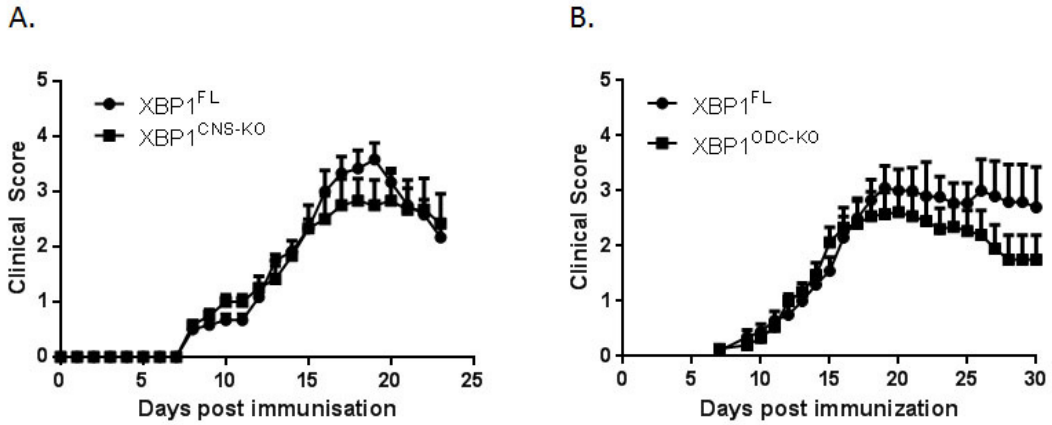

Figure 3. A. Clinical disease scores of XBP^{FL}(n=6) and $XBP^{CNS\text{-}KO}$ (n=6) littermate mice after immunization with MOG peptide. B. Clinical disease scores of XBP^{FL}(n=10) and $XBP^{ODC\text{-}KO}$ (n=15) littermate mice after immunization with MOG peptide. Results are presented as mean values ± s.e.m.

1.4. XBP1 in cuprizone-induced demyelination

Brain demyelination can be induced through the administration of cuprizone in chow diet. Although little is known of the mechanisms by which cuprizone induces CNS demyelination and inflammation, cuprizone administration induces activation of ER stress response genes (Figure 4).

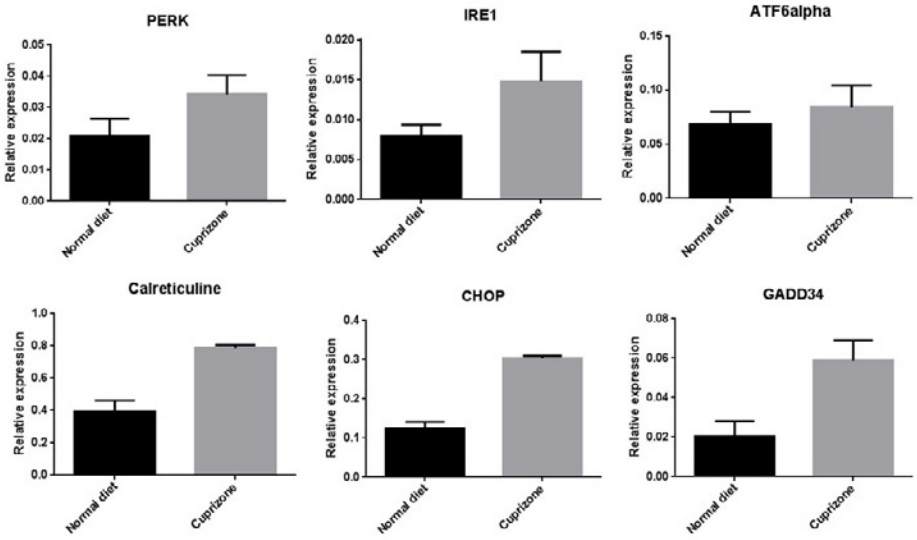

Figure 4. Cuprizone-induced expression of UPR genes. Expression of ER stress markers was assessed through qPCR on corpus callosum lysates isolated from wild-type C57BL/6 mice either or not treated with cuprizone for 5 weeks. Results are presented as mean values ± s.d.

Next, XBP1^{CNS-KO} mice and control littermates were given cuprizone for 5 weeks and degree of demyelination and inflammation was assessed in corpus callosum. Wild-type mice show a near complete demyelination of the corpus callosum, as expected, in contrast to XBP1^{CNS-KO} mice which were significantly protected from cuprizone-induced demyelination. On brain histology, XBP1^{CNS-KO} mice also showed a significant reduction in microgliosis and astrogliosis, and a higher number of mature oligodendrocytes (Figure 5). Since XBP1 deficiency was shown to be protective in a mouse model of ALS through enhanced clearance of protein aggregates by autophagy (Hetz et al., 2009), the protection of XBP1^{CNS-KO} mice in the model of cuprizone-induced demyelination may also depend on an increased autophagy of dysfunctional mitochondria protecting oligodendrocytes from cell death. XBP1 deficiency may also protect oligodendrocytes from apoptosis involving the pro-apoptotic proteins BAX, BAK, and ASK1-interacting protein 1 (AIP1) (Hetz et al., 2006). This hypothesis may also explain the mild protection of XBP1^{ODC-KO} mice in EAE. These studies are currently under investigation.

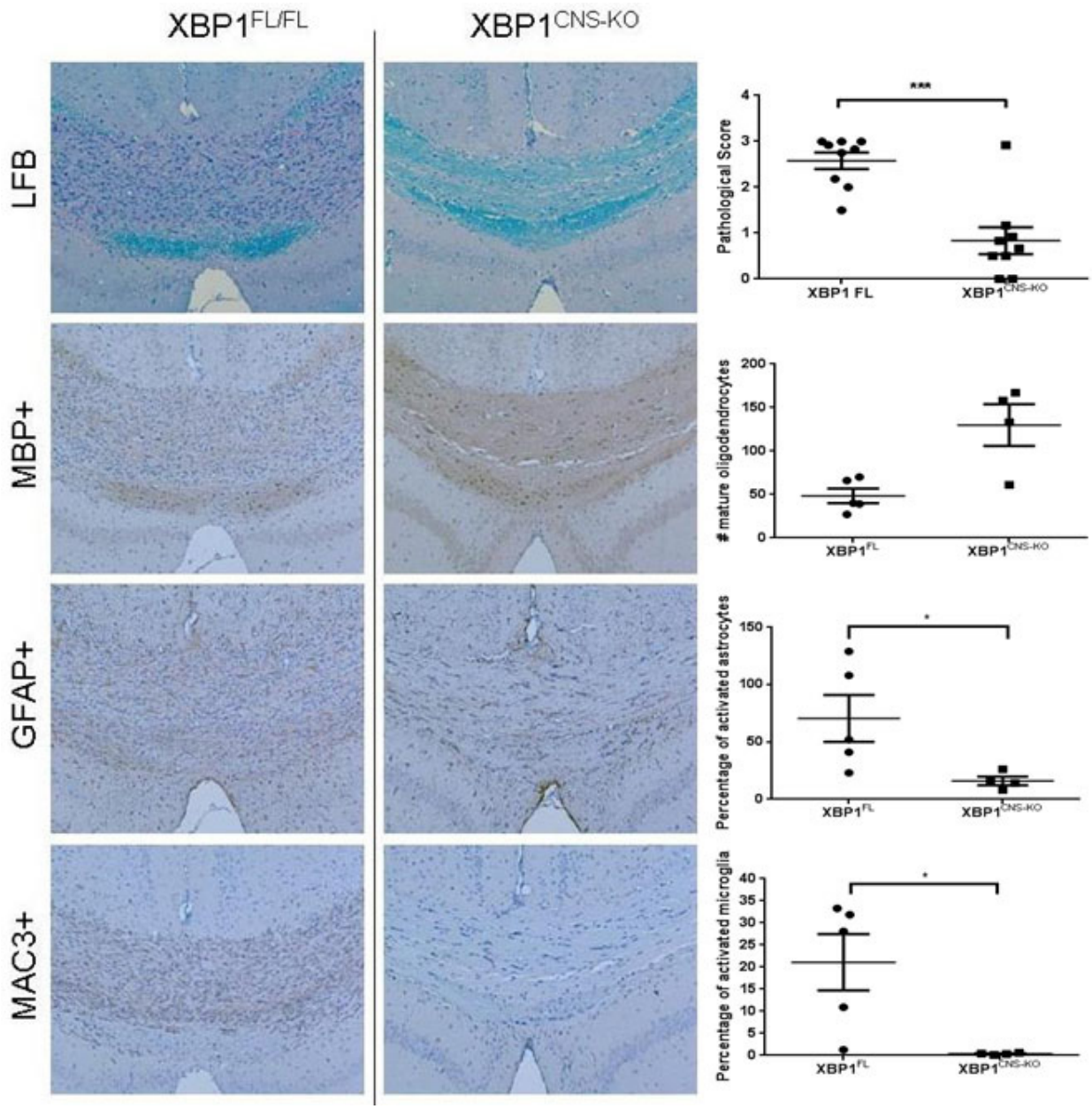

Figure 5. Cuprizone-induced demyelination is reduced in XBP1^{CNS-KO} mice. Representative pictures of coronal sections of the corpus callosum (left) and quantification (right) for degree of demyelination (LFB), oligodendrocyte numbers (MBP), astrogliosis (GFAP) and microgliosis (Mac3) in XBP1^{CNS-KO} mice and XBP1^{FL} littermates after 5 weeks of cuprizone treatment.

2. Future studies

During the second year of the project period, we will further elaborate on the phenotype of the CNS-specific XBP1 knockout mice in the model of cuprizone-induced inflammation and CNS demyelination and dissect the contribution of ER stress in the different CNS cell types involved. Next, new mouse lines with specific defects in IRE1α– and PERK-dependent UPR signalling and autophagy in CNS cell types have been generated allowing their study in both EAE and cuprizone models of MS. Also, *in vitro* biochemical studies using primary cultures isolated from the respective knockout mice will be used in order to establish the mechanisms by which ER stress and autophagy control inflammatory responses. Finally, our different brain-specific knockout mice will also be subjected to other models of CNS inflammation and pathology, such as to a model of cerebral ischemia. Although there is strong evidence that inflammation contributes to the pathology of cerebral ischemia, very little is known of the contribution of ER stress and autophagy to the inflammatory processes associated with the disease.

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Mechanisms of neurogenesis and cortical development, and implications for brain diseases.

State of the art and objectives.

The cerebral cortex is one of the most important structures in our brain: to a large extent it is considered to underlie what we are as a species, and as individuals. In correlation with its elaborate functions, the cerebral cortex displays multiple levels of complexity. Its surface is parcellated into numerous areas characterized by specific patterns of connectivity, and thereby underly selective functional modalities such as motor control, vision, or language for instance (Grove and Fukuchi-Shimogori, 2003; Sur and Rubenstein, 2005). Each cortical area is further divided through its depth into distinct cortical layers, each of which consists of neurons displaying stereotypic patterns of input and output (Molyneaux et al., 2007). Within each layer, several classes of neurons can be further distinguished on the basis of their gene expression, neurotransmission, morphology and/or connectivity. Overall, the cerebral cortex can thus be thought of as a collection of hundreds of different types of neurons, and this diversity is at the core of its powerful computational capacities.

The mechanisms of neurogenesis and cortical development have important implications for our understanding of pathological brain development and in the long run for the rational design of replacement therapies of neurological conditions, many of which strike the cerebral cortex.

We previously uncovered an intrinsic pathway of cortical neurogenesis, whereby pluripotent stem cells (embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC)), whether of mouse or human origin, efficiently generate neurons that share most molecular, cellular and functional landmarks of pyramidal neurons of the cerebral cortex (Anderson and Vanderhaeghen, 2014; Espuny-Camacho et al., 2013; Gaspard et al., 2009; Gaspard et al., 2008; van den Ameele et al., 2014). 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 neurogenesis and cortical development in health and disease.
The results obtained so far are detailed below:

1. Mechanisms of neurogenesis, and implication in brain cancer.
2. Exploring new ways to repair the diseased cortex.
3. Modelling human neurodevelopment diseases.

1. Mechanisms of neurogenesis, and implication in brain cancer.

Using our *in vitro* model of corticogenesis, we recently identified BCL6, a transcriptional repressor and B cell oncogene, as a key factor to promote the generation of pyramidal neurons in the cortex (Tiberi et al., 2012). To follow up on these results, we have now explored additional roles of BCL6 in brain development, focusing on the cerebellar cortex, where we found BCL6 to be expressed prominently during postnatal neurogenesis.
We first found that BCL6 was necessary and sufficient to induce the differentiation of granule neuron precursors (GNP) into cerebellar granule neurons, the most abundant neurons of the brain. Then using a combination of cell and molecular approaches, we discovered that BCL6 promoted GNP differentiation through the repression of Gli1/2 transcription factors, the main effectors of the Sonic Hedgehog (SHH) pathway, which is key for the expansion and self-renewal of GNP (Hatten and Roussel, 2011).

Given that the SHH pathway is frequently mutated and overactivated in specific forms of medulloblastoma (MB) (Olson, 2014), the most frequent pediatric brain tumour, we then explored whether and how BCL6 could be implicated in this form of cancer, using ad how mouse models. We thus found that BCL6 overexpression was sufficient to block the initiation and growth of MB in a mouse model of SHH-dependent MBn which was confirmed in an *in vitro* model of human SHH medulloblastoma cell line. Besides and importantly, we found that mouse defective in BCL6 and p53 function were much more prone to develop MB, thus qualifying BCL6 as a bona finde tumour suppressor.

Our findings identify BCL6 as a potent repressor of the SHH pathway in normal and oncogenic neural development, with direct relevance for human SHH medulloblastoma. These findings thus identify new therapeutic and diagnostic targets for this brain tumour, and illustrate how the same gene can act as an oncogene (in B lymphocytes) and an oncosuppressor (in granule neuron precursors), depending on the cellular context.

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

- A BCL6/BCOR/SIRT1 complex Triggers Neurogenesis and Suppresses Medulloblastoma by Repressing Sonic Hedgehog Signalling.
  Tiberi L, Bonnefont J, van den Ameele J, Le Bon S, Herpoel A, Bilheu A, Baron BW, and *Vanderhaeghen P*.
  Cancer Cell (2014) 26, 797-812.
  Preview and Featured Article in Cancer Cell.
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2. Exploring new ways to repair the diseased cortex.

While pluripotent stem cell-derived neurons constitute an attractive source for replacement therapies, it remains unclear whether they could be useful for cortical diseases. 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 adult 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, mouse ESC-derived cortical progenitors and neurons (generated following (Gaspard et al., 2009), which mostly display an identity of neurons of the visual cortex (Gaspard et al., 2008), 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 (motor) 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. Finally we assessed 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 extracellular recordings. These studies revealed 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 findings demonstrate that transplantation of mouse ESC-derived neurons of appropriate cortical areal identity can contribute to the reconstruction of an adult damaged cortical circuit. We are now following up on these findings by testing whether and how human PSC-derived neurons can integrate and contribute to reconstruction of damaged cortical circuits in the adult mouse, using our recently established protocols of human corticogenesis (Espuny-Camacho et al., 2013), as well as new improved methos of intracortical transplantation (Nagashima et al., 2013).

This set of results is part of the following publications:

- Area-specific reestablishment of damaged circuits in the adult cerebral cortex by cortical neurons derived from mouse embryonic stem cells.
  Michelsen KA , Acosta-Verdugo P, Benoit-Marand M, Espuny-Camacho I, Gaspard N, Saha B, Gaillard A , and *Vanderhaeghen P*.
  Neuron* (2015), *in press.
  IF: 16.7
- Novel and robust transplantation reveals the acquisition of polarized processes by cortical cells derived from mouse and human pluripotent stem cells.
  Nagashima F, Suzuki IK, Shitamukai A, Sakaguchi H, Iwashita M, Kobayashi T, Tone S, Toida K, *Vanderhaeghen P*, Kosodo Y.
  ***Stem Cells Dev.* (2014) 23 (18):2129-42.**
  IF: 3.9

3. Modelling human neurodevelopment diseases.

Based on our previous results showing that corticogenesis can be faithfully recapitulated not only from human ESC but also iPSC (Espuny-Camacho et al., 2013), we have started to use this technology to model cortical neurodevelopmental diseases for which the underlying causes remain poorly known, and where human-specific developmental mechanisms may be implicated. We have implemented iPS models of non syndromic primary microcephaly (MCPH) (Bond and Woods, 2006; Kaindl et al., 2010), a rare autosomal recessive condition where patients display mental retardation and a small brain size, in particular of the cerebral cortex. Mutations in a dozen genes have been implicated in MCPH, most of which encode centrosomal proteins, but the mechanisms by which their disruption causes defects in corticogenesis remain unclear.

Using standard iPS reprogramming technology (Takahashi et al., 2007) we have generated pluripotent cell lines from skin fibroblasts of patients affected by 3 microcephaly gene mutations: MCPH2 (WDR62); MCPH4 (CASC5), and MCPH5 (ASPM), as well as from control unaffected subjects (Espuny-Camacho et al., 2013).

We have used these lines to try and decipher the developmental mechanisms underlying primary microcephaly. Cortical-like progenitors derived from microcephaly or control patient iPSC have been examined for various key parameters of neurogenesis, including proliferation and survival of progenitors, patterns of symmetrical or asymmetrical division, as well as neuronal production and specification. Doing so we obtained first exciting results on the mechanisms underlying the pathogenesis of microcephaly caused by mutations in ASPM. We found that ASPM-deficient cortical progenitors undergo precocious neurogenesis, display abnormal orientation of the mitotic spindle pole, and defective acquisition of forebrain/cortical identity. Intriguingly, the defects could be linked to Wnt signalling in ASPM deficient neuroepithelial cells, as the phenotypes of ASPM mutated cells were partially rescued by Wnt inhibition, while Wnt overactivation in control cells could result in ASPM-mutant like phenotypes. These data reveal

how ASPM mutations may lead to decreased human brain size, and uncover a surprising link between this pericentrosomal protein and Wnt signalling during human neurogenesis.
Part of this work is now part of a manuscript in preparation.

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Most significative publications from work performed in 2014

- Specific reestablishment of damaged circuits in the adult cerebral cortex by cortical neurons derived from mouse embryonic stem cells.
  Michelsen KA , Acosta-Verdugo P, Benoit-Marand M, Espuny-Camacho I, Gaspard N, Saha B, Gaillard A , and **Vanderhaeghen P**.
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- Restoration of Progranulin Expression Rescues Cortical Neuron Generation in an Induced Pluripotent Stem Cell Model of Frontotemporal Dementia.
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  IF: ND
- Thinking out of the dish: what to learn about cortical development using pluripotent stem cells.
  van den Ameele, J., Tiberi, L., V**anderhaeghen, P*.**, and Espuny-Camacho, I.
  **corresponding author.*
  Trends Neurosci. (2014), 37, 334-342.
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- Novel and robust transplantation reveals the acquisition of polarized processes by cortical cells derived from mouse and human pluripotent stem cells.
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  Stem Cells Dev. (2014) 23(18):2129-42.
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- Cortical neurogenesis from pluripotent stem cells: complexity emerging from simplicity.
  Anderson, S., and ***Vanderhaeghen, P.***
  Curr Opin Neurobiol (2014) 27C, 151-157.
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