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

Verslag – Rapport – Bericht – Report

2015

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

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Geneeskundige Stichting Koningin Elisabeth
2015
Inleiding verslag activiteiten van de GSKE – FMRE

Het jaar 2015 is het tweede jaar van de driejaarlijkse (2014-2015-2016) ter ondersteuning van de projecten voorgelegd aan de Geneeskundige Sticvhting Koningin Elisabeth (G.S.K.E.).

Elk onderzoeksteam stelt in dit document de vorderingen voor van de werkzaamheden gedurende het jaar 2015.

Dankzij een goed beheer en de gunstige algemene financiële situatie, hebben de ploegen kunnen genieten van dezelfde onderzoekskredieten als die van het voorgaande jaar. Dit werd beslist tijdens de laatste bestuursvergadering in december 2014.

Een regelmatige controle door de diensten van de respectievelijke universiteiten en door de administratie van de G.S.K.E., heeft aangetoond dat alle door de onderzoekteams gemaakte uitgaven kaderen in de projecten.

Tijdens de traditionele Academische zitting, uitreiking van de prijzen, in de maand mei, werden na een beslissing van de Raad van Bestuur op voorstel van het wetenschappelijk comité 3 laureaten bekroond. Het betreft:
- Dr. Pierre Vanderhaeghen (ULB): Prijs Solvay
- Prof. dr. Geert van Loo (UGent): Prijs CBC Bank
- Prof. dr. Serge N. Schiffmann (ULB): Prijs Janine en Jacques Delruelle

Tijdens deze zitting luisterden de talrijke aanwezigen onder andere naar een boeiende uiteenzetting door Professor Jean-Jacques Cassiman (KU Leuven) over de recentste ontwikkelingen in de genetica.

Onze Erevoorzitster, Prinses Astrid kon, ondanks Haar drukke agenda, zich twee halve dagen vrijmaken om een bezoek te brengen aan de labo's van de onderzoeksploegen die gesteund worden door de G.S.K.E., meer bepaald deze van Professor Fadel Tissir (UCL) en Professor dr. Geert van Loo (UGent)

Bij elk bezoek wordt Ze begroet door de lokale academische en politieke overheden en de leden van de onderzoeksteams. Deze bezoeken zijn een gelegenheid voor ontmoetingen en uitwisselingen op hoog niveau.

Niets zou er gebeurd zijn tijdens het jaar, indien de Raad van Bestuur, met kennis van zaken, zijn advies niet zou gegeven hebben betreffende het administratief beheer en de toewijzing van de financiële middelen. Bij deze dank ik hen officiëel hiervoor.

Een speciale erkenning gaat naar H.K.H. Prinses Astrid. Haar aandacht, Haar ijver en Haar actieve deelname aan alle activiteiten van de Stichting betekenen voor het Wetenschappelijk Comité en de onderzoekers een duidelijke en waardevolle steun.

Prof. em. dr. Baron de Barsy,
wetenschappelijk directeur
Brussel, 30 december 2015

Fondation Médicale Reine Elisabeth
2015
Introduction rapport d'activités de la FMRE – GSKE

L'année 2015 est la deuxième année de la triennale (2014-2015-2016) de soutien des projets soumis à la Fondation Médicale Reine Elisabeth (F.M.R.E.).

Chaque équipe de recherche présente, dans ce document, l'état d'avancement des travaux effectués au cours de 2015.

Les équipes ont pu bénéficier, grâce à la bonne gestion et à la situation financière générale, de crédits identiques à ceux de l'année précédente. Une décision dans ce sens avait été prise au cours du dernier conseil d'administration de décembre 2014.

Un contrôle régulier, par les services des universités respectives et par l'administration de la F.M.R.E., a pu constater que toutes les dépenses engagées par les équipes de recherches correspondent aux projets.

La séance traditionnelle du mois de mai, séance de remise des prix, a permis de récompenser 3 lauréats, suite à la décision du conseil d'administration, qui a entériné le classement proposé par le comité scientifique. Il s'agit de :
- Dr. Pierre Vanderhaeghen (ULB) : Prix Solvay
- Prof. dr. Geert van Loo (UGent) : Prix CBC Banque
- Prof. dr. Serge N. Schiffmann (ULB): Prix Janine en Jacques Delruelle

Au cours de cette séance, l'assemblé nombreuse a pu entendre ,en outre, un exposé passionnant du Professeur Jean-Jacques Cassiman (KU Leuven) sur les progrès récents en génétique.

Notre présidente d'honneur, la Princesse Astrid a pu, malgré un horaire chargé, consacrer deux demi journées à la visite de laboratoires de recherches soutenus par la FMRE, celui du Professeur Fadel Tissir à l'UCL et celui du Professeur Geert Van Loo à la UGent.

A chaque visite Elle fut accueillie par les autorités locales, académique et politiques, et les membres des équipes de recherches. Ces visites sont l'occasion de rencontres et d'échanges de haut niveau.

Rien ne se passerait au cours de l'année, si le conseil d'administration ne donnait pas son avis éclairé, concernant la gestion administrative et l'attribution des moyens financiers. Qu'il en soit ici officiellement remercié.

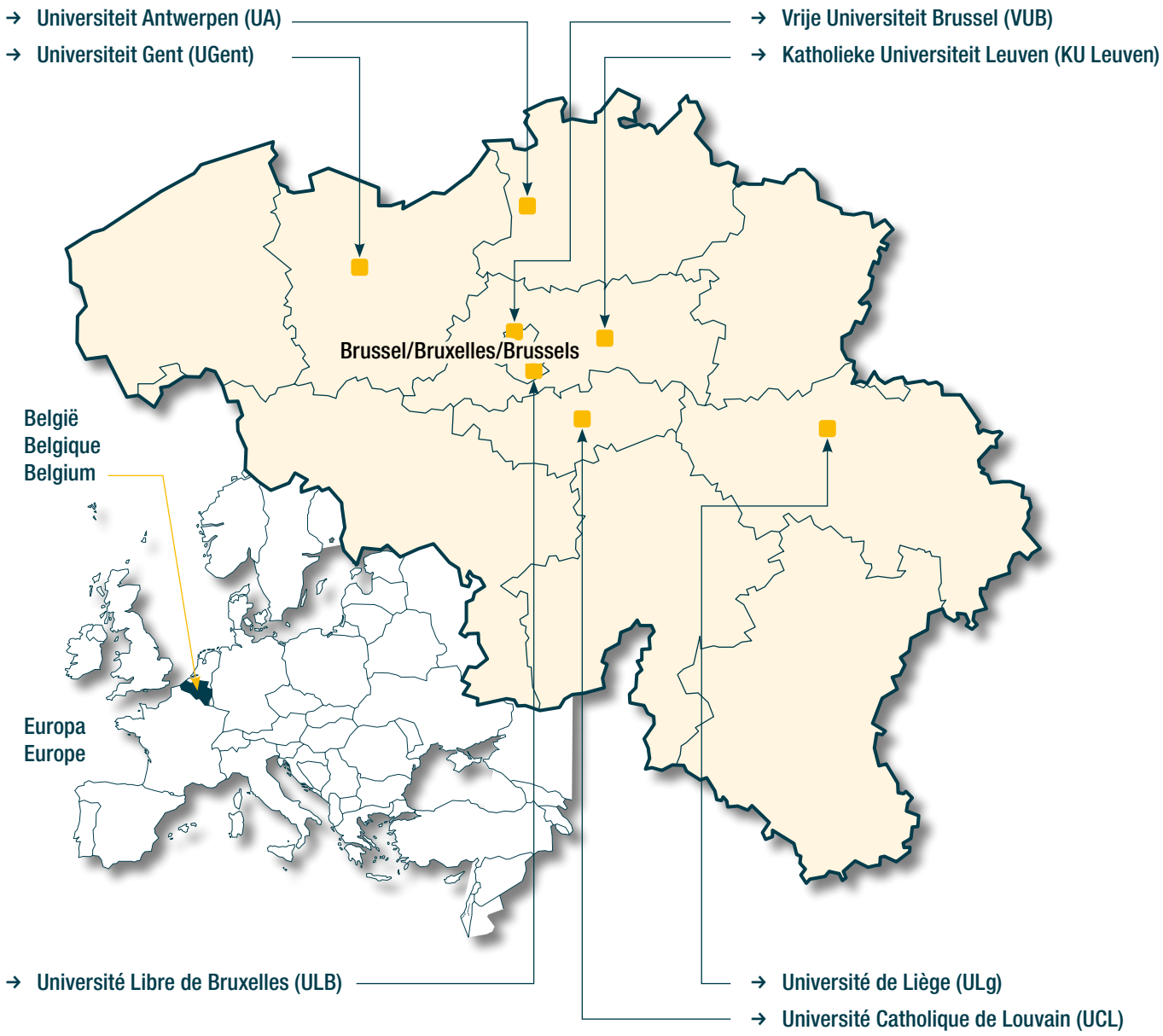
Une reconnaissance toute particulière s'adresse la Princesse Astrid. Son attention soutenue, sa diligence et sa participation active à toutes les activités de la Fondation sont pour le comité scientifique et la communauté des chercheurs un soutien évident et précieux.

Prof. em. dr. Baron de Barsy,
directeur scientifique
Bruxelles, 30 décembre 2015

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 (2015)

Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
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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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Table of contents

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

1. RESEARCH PROGRAM

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's 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. Reduction of CYFIP1 leads to spine dysmorphogenesis and that the CYFIP1 interactome is enriched in proteins associated to SCZ, ASD, ID and EPY.
Deletions and duplications of the locus containing CYFIP1 have been identified in several neurological disorders. 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 affecting the CYFIP1 signaling pathways. Finally, we aim at setting the ground for appropriate pharmacological therapies for these heterogeneous groups of disabilities such as ASD, FXS, MDD, SCZ, AD.

2. ACHIEVEMENTS

Achievement 1: Human sample collection. During the first year of this grant support we gathered brain specimens from publicly available biobanks as the Stanley Medical Research Institute and the Maryland Brain biobanks. 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 (Belgium) and at Tor Vergata Hospital (Italy). Preliminary data on human cingulate cortex showed that two CYFIP1 interactors are downregulated in patients with Schizophrenia (n=14) and Bipolar Disorder (n=13). Protein levels were analyzed blindly by Western blotting and normalized to Vinculin. *P-value<0.05. One-way ANOVA followed by post-hoc Bonferroni correction. This first data set showed that proteins that are part of the CYFIP1 interactome are down-regulated in human postmortem brains of patients with BD and SCZ, two typical examples of synaptopathies.

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. Sequence analysis revealed that the 15q11.2 del was inherited from the mother and the SNV from the father. Based on the crystal structure of WRC, molecular modeling of the alteration introduced by the CYFIP1 suggested a loss of electrostatic interaction between CYFIP1 and three key components of its interactome namely NCKAP1, WAVE, ABI2.

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 mut in HEK293T cells, we observed that the association of CYFIP1 with components of the WRC was reduced in cells transfected with mutated CYFIP1. CYFIP1 mut 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 CYFIP1 mut in these pathways in the patient's cells. Importantly, patient's fibroblasts showed an increase of newly synthetized proteins consistent with a role of CYFIP1 in negatively regulating protein translation (Napoli et al., 2008; De Rubeis et al., 2013).

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. Such a decrease is also supported by a reduction in phalloidin intensity. Patient's cells displayed as well a difference in the cell morphology, appearing more elongated (ratio major/minor axis). These deficits are CYFIP1 dependent because the reintroduction of YFP-CYFIP1 in patient's cells reverted these cellular phenotypes. These results unequivocally implicated CYFIP1 dysfunction in the observed cellular defects.

Achievement 5: In vivo characterization of the R826Q mutation in *CYFIP1* using a Drosophila model. Because of the genetic heterogeneity between patients with ID, it has been a major challenge to comprehensively study genetic causes of ID. The fruit fly **Drosophila** has emerged as a very powerful organism for such endeavors. Using the genetic advantages of fly model, we generated flies expressing the R826Q mutation in the *CYFIP1* gene and characterized its function *in vivo*. The last year we performed the cloning of the R826Q mutation and we generated transgenic flies expressing the mutation under spatiotemporal conditions. We show that expression of the R826Q mutation in the *CYFIP1* gene recapitulates in Drosophila features of patients with ID and ASD such as hyperactivity and social interaction deficits.

Achievement 6: CYFIP1, eIF4E and FMR1 mRNA and protein levels are not altered in a group of patients with ASD. CYFIP1, eIF4E and FMR1 mRNA and protein levels were analyzed on a larger number (compared to the initial study during the first year) of from non-syndromic ASD aged 6-12 (n=15) and control (n=13) by quantitative western blotting and real time qPCR respectively. This analysis revealed that the levels of CYFIP1, eIF4E and FMR1 are not significantly altered in this subgroup of ASD patients compared to healthy controls. Importantly, we observed the a specific signaling pathway regulating FMRP functions is affected in a specific group of patients with ASD. We are currently correlating this molecular signature to the clinical features of these patients with ASD (Battan, Rosina, Pacini et al., in preparation).

3. 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. We are actively collaborating with clinicians and human geneticists at UZ Leuven (Prof. Koenrad De Vrient and Prof. Hilde Peeters) and at Tor Vergata Hospital (Prof. Paolo Curatolo). We have also initiated a new collaboration to study additional CYFIP1 mutations associated to ASD with Prof Pierre Billuart and Prof Thierry Bienvenu at Institut Cochin, INSERM Paris. Finally, we have a fruitful collaboration with the microscope imaging facility of the Center of Human Genetics, KU Leuven (Light Microscopy & Imaging Network, LiMoNe) to study actin remodeling and analysis.

4. RELEVANCE

The work we have performed during the last 2 years with the support of the FMRE helped us to increase the current knowledge of CYFIP1 distribution and function/s in brain using a mouse and fly model and, importantly, human cells from 2 patients with mutation in the CYFIP1 gene. We are aiming at understanding how a single molecule, CYFIP1, affecting two key cellular processes as protein synthesis and actin remodeling if mutated or reduced causes ASD. Our data have so far identified impaired cellular processes that might explain the neuronal deficits in the two identified patients. We therefore think that CYFIP1 could be the entry key to a better understanding of ASD. We hope that the *Fondation Médical Reine Elisabeth* continues to support our research.

5. PUBLICATIONS UNDER THE SUPPORT OF FMRE (2014-2016)

1. Panja D, Kenney JW, D'Andrea L, Zalfa F, Vedeler A, Wibrand K, Fukunaga R, **Bagni C**, Proud CG and Bramham CR (2014). "Sustained BDNF-TrkB signaling to MNK mediates two-stage translational control of LTP consolidation in the dentate gyrus in vivo" ***Cell Reports***, 9:1430-45. doi: 10.1016/j.celrep.2014.10.016. Epub 2014 Nov 6.
2. Pasciuto E, Borrie SC, Kanellopoulos AK, Santos AR, Cappuyns E, D'Andrea L, Pacini L, **Bagni C.** (2015). Autism Spectrum Disorders: Translating human deficits into mouse behavior. Neurobiol Learn Mem. Jul 26. pii: S1074-7427(15)00134-3. doi: 10.1016/j.nlm.2015.07.013. [Epub ahead of print] Review. PubMed PMID:26220900.
3. Di Marino D, D'Annessa I, Tancredi H, **Bagni C** and Gallicchio E (2015). A Unique Binding Mode of the Eukaryotic Translation Initiation Factor 4E for Guiding the Design of Novel Peptide Inhibitors. Protein Science. 2015 Sep;24(9):1370-82. doi: 10.1002/pro.2708.
4. Di Marino D, Chillemi G, De Rubeis S, Tramontano A, Achsel T* and B**agni C*.** (2015) MD and docking studies reveal that the functional switch of CYFIP1 is mediated by a butterfly-like Motion. Journal of Chemical Theory and Computation, 2015 April; 11,3401-3410; doi: 10.1021/ct500431h. * corresponding authors.
5. Santos AR, Kanellopoulos A and **Bagni C** (2014). Learning and Behavioral Deficits Associated with Absence of the Fragile X Mental Retardation Protein: what a fly and mouse models can teach us. Learning and Memory, 21: 543-555.
6. Di Marino D, Achsel T, Lacoux C, Falconi M, **Bagni C.** (2014). Molecular dynamics simulations show how the FMRP Ile304Asn mutation destabilizes the KH2 domain structure and affects its function. J. Biomol. Struct. Dyn. 32:337-50.

In preparation

– Ana Rita Santos*, Vittoria Mariano*, Alexandros Kanellopolous, Sarah Borrie, Veerle De Wolf, Esperanza Fernandez, Daniele Di Marino, Tilmann Achsel, Sebastian Dupraz, Frank Bradke, Hilde Van Esch, Koenraad Devriendt, Hilde Peeters, Pierre Billuart, Thierry Bienvenu and Claudia Bagni (2016). "Mutations in the human ASD gene CYFIP1 affect cellular homeostasis and behavior". *In preparation.*
– Barbara Battan*, Eleonora Rosina*, Laura Pacini*, Paolo Curatolo and Claudia Bagni (2016). "A molecular signature modulating FMRP activity is identified in a group of patients with non-syndromic ASD". In preparation.

6. TEAM KEY PUBLICATIONS (5 selected from 25 publications over the last five years)

1. Pasciuto E, Ahmed T, Wahle T, Gardoni F, D'Andrea L, Pacini L, Jacquemont S, Tassone F, Balschu D, Dotti CG, Vegh Z, D'Hooge R, Müller U, Di Luca M, De Strooper B and **Bagni C** (2015). Dysregulated ADAM10-mediated processing of APP during a critical time-window leads to synaptic deficits in fragile X syndrome. ***Neuron***, 87: 382-398.
2. 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.
3. Pasciuto E and **Bagni C** (2014). "FMRP mRNA Targets and Associated Human Diseases" ***Cell*** *SnapShot*, 158 (6): 1446-1446.
4. Pasciuto E and **Bagni C** (2014). "FMRP Interacting Proteins" ***Cell*** *SnapShot* 159 (1):218-218.
5. 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.

Geneeskundige Stichting Koningin Elisabeth
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Progress report of the research group of

Prof. dr. Marc Cruts, PhD

Universiteit Antwerpen (UA)

Principal investigator

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Co-investigators

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Neurodegenerative Brain Diseases Group
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Laboratory for Neurogenetics
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Table of contents

Integrative -Omics Studies of Frontotemporal Lobar Degeneration and Related Diseases

1. Specific Aims

Behavioral variant frontotemporal dementia (bv-FTD), semantic dementia (SD), and progressive non-fluent 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 known FTD genes
2. Identification of novel causal genes for FTLD
3. Identification of genes modifying onset age in FTLD

In 2015, progress was made in objectives 1 and 2, as detailed below.

2.1. C9orf72 repeat expansions

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., 2012). Because diverse disease mechanisms were hypothesized to be associated with pathological repeat expansions (Cruts, et al., 2013), we further studied the mechanism by which the *C9orf72* repeat expansion causes disease.

We investigated the involvement of a toxic gain-of-function mechanism by studying the effect of repeat expansion size 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. We assessed the effect of G4C2 expansion size on onset age, the role of anticipation and the effect of repeat size on methylation and *C9orf72* promoter activity. G4C2 expansion sizes varied in blood between 45 and over 2100 repeat units with short expansions (45–78 units) present in 5.6% of 72 index patients with a pathological expansion. Short expansions co-segregated with disease in two families. Detailed analysis of the subject with a short expansion in blood indicated mosaicism in brain and showed the same pathology as those with a long expansion. Further, we provided evidence for an association of G4C2 expansion size with onset age ($P < 0.05$) (**Figure 1**) most likely explained by an association of methylation state of the 5' flanking CpG island and expansion size in blood ($P < 0.0001$) and brain ($P < 0.05$) (**Figure 2**). In several informative *C9orf72* parent–child transmissions, we identified earlier onset ages, increasing expansion sizes and/or increasing methylation states (P=0.0034) of the 5' CpG island, reminiscent of disease anticipation. Also, intermediate repeats of 7–24 units showed a slightly higher methylation degree ($P < 0.0001$) and a decrease of *C9orf72* promoter activity ($P < 0.0001$) compared with normal short repeats of 2–6 units. Decrease of transcriptional activity was even more prominent in the presence of small deletions flanking G4C2 ($P < 0.0001$). Here we showed that increased methylation of CpGs in the *C9orf72* promoter may explain how an increasing G4C2 size leads to loss-of-function without excluding repeat length-dependent toxic gain-of-function. These data provide insights into disease mechanisms and have important implications for diagnostic counseling and potential therapeutic approaches (Gijselinck, et al., 2015a).

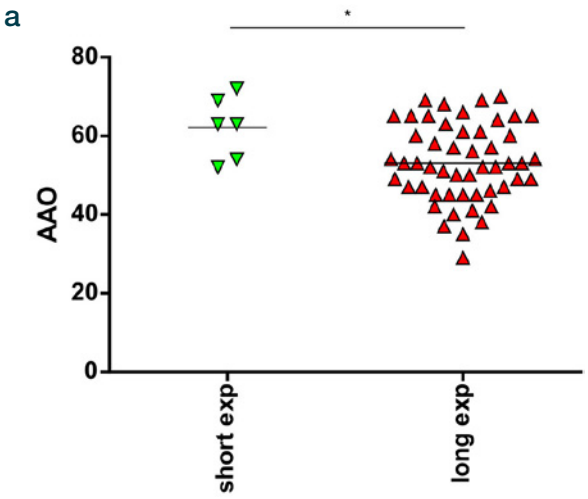

Figure 1. Association of G_4C_2 size with onset age of disease. Comparison of age at onset (AAO) between patients with a short expansion (<80 units) and patients with a long expansion (>80 units) ($P < 0.05$) (Gijselinck, et al., 2015a).

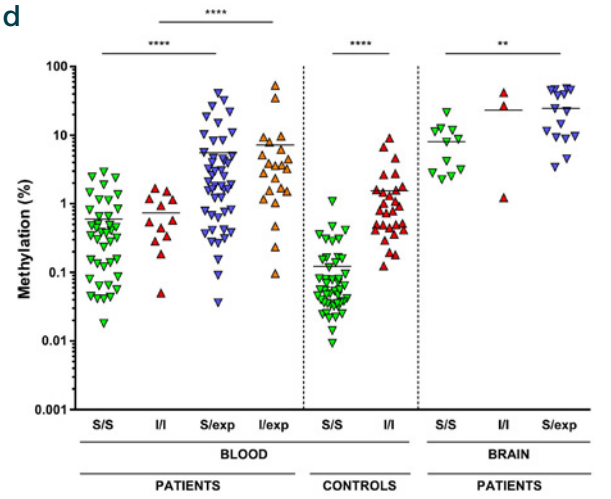

Figure 2. Association of G_4C_2 size with DNA methylation of the 5' flanking CpG island. *Hha*I MSRE-qPCR results of the 5' flanking CpG island are presented for expansion carriers (S/exp and I/exp) versus patients without expansion with short/short (S/S) and intermediate/intermediate (I/I) genotype stratified for normal short (S) or intermediate (I) repeat length of the normal alleles, and for controls with S/S and I/I genotype (Gijselinck, et al., 2015a).

2.2. GRN missense mutations

Progranulin (GRN) is a multifunctional secreted growth factor involved in various important cellular functions and loss-of-function mutations are a major cause of FTLD with TDP-43 positive pathology. The majority of FTLD-related *GRN* mutations are nonsense mutations resulting in reduced *GRN* expression through a haploinsufficiency mechanism. However, non-synonymous missense mutations, scattered over all *GRN* exons, have also been described as risk factors to develop other neurodegenerative brain diseases. While some missense variants alter the secretion efficiency of GRN or the conversion of the GRN precursor protein into individual granulin peptides, the pathogenic nature of most GRN variants remains to be determined. We identified a double missense mutation in *GRN* leading to amino acid changes p.D33E and p.G35R in a patient from Turkish origin. Genetic analyses of the transmission pattern in five offspring suggested a transmission of the mutation in cis. Biochemical and cell biological analysis of the double mutation and two additional, earlier described, patient-specific GRN missense mutations (p.C105R and p.V514M) revealed a reduced transport of the GRN p.D33E/G35R and p.C105R proteins through the secretory pathway leading to lowered levels of secreted GRN. Furthermore, we showed that loss of a conserved cysteine residue affects proper protein folding, resulting in reduced secretion and altered proteolytic processing by neutrophil elastase and proteinase 3. Our data thus indicated that GRN mutations may affect GRN homeostasis at multiple levels (Kleinberger, et al., 2015).

3. Novel Causal FTD Genes

3.1. *TBK1*

Mutations in the TANK-binding kinase 1 gene (*TBK1*) were identified in the FTD-ALS spectrum of diseases. We assessed the genetic contribution of *TBK1* in Belgian FTD and ALS patient cohorts containing a significant part of genetically unresolved patients. We sequenced *TBK1* in a hospital-based cohort of 482 unrelated patients with FTD and FTD-ALS and 147 patients with ALS and an extended Belgian FTD-ALS family DR158. We followed up mutation carriers by segregation studies, transcript and protein expression analysis, and immunohistochemistry. We identified 11 patients carrying a loss-of-function (LOF) mutation resulting in an overall mutation frequency of 1.7% (11/629), 1.1% in patients with FTD (5/460), 3.4% in patients with ALS (5/147), and 4.5% in patients with FTD-ALS (1/22). We found 1 LOF mutation, p.Glu643del, in 6 unrelated patients segregating with disease in family DR158. Of 2 mutation carriers, brain and spinal cord was characterized by TDP-43-positive pathology. The LOF mutations including the p.Glu643del mutation led to loss of transcript or protein in blood and brain. *TBK1* LOF mutations are the third most frequent cause of clinical FTD in the Belgian clinically based patient cohort, after *C9orf72* and *GRN*, and the second most common cause of clinical ALS after *C9orf72*. These findings reinforce that FTD and ALS belong to the same disease continuum (Gijselinck, et al., 2015b).

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5. Activity Report

5.1. Oral Presentations at International Meetings

- Van Broeckhoven,C.: American Society of Human Genetics Annual Meeting 2015, Baltimore, USA, October 6-10, 2015. Loss-of-function mutations in TBK1 are a frequent cause of frontotemporal dementia and amyotrophic lateral sclerosis in Belgian and European cohorts.
- Cruts, M.: American Society of Human Genetics Annual Meeting 2015, Baltimore, USA, October 6-10, 2015. The C9orf72 repeat expansion modulates onset age of FTD-ALS through increased DNA methylation and transcriptional downregulation.
- Van Broeckhoven,C.: Alzheimer's Association International Conference 2015, Washington, USA, July 18-23, 2015. Loss-of-Function mutations in TBK1 are frequently associated with frontotemporal lobar degeneration in a Belgian patient cohort.
- Philtjens, S: EMBO | EMBL Symposium Mechanisms of Neurodegeneration, Heidelberg, Germany, June 14-17, 2015. Novel missense mutations in the vacuolar protein sorting 13 homolog C gene are associated with decreased endogenous protein expression in frontotemporal lobar degeneration.

5.2. Poster Presentations at International Meetings

- Wauters, E.: EMBO | EMBL Symposium Mechanisms of Neurodegeneration. Heidelberg, Germany, June 14-17, 2015. An integrative approach to identify onset age modifier genes in an extended Belgian GRN founder family.
- Philtjens, S: EMBO | EMBL Symposium Mechanisms of Neurodegeneration, Heidelberg, Germany, June 14-17, 2015. Novel missense mutations in the vacuolar protein sorting 13 homolog C gene are associated with decreased endogenous protein expression in frontotemporal lobar degeneration.

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

6.1. Articles in International Journals

- **Gijselinck,I.**, Van Mossevelde,S., van der Zee,J., Sieben,A., Engelborghs,S., De Bleecker,J., Ivanoiu,A., Deryck,O., Edbauer,D., Zhang,M., Heeman,B., Bäumer,V., Van den Broeck,M., Mattheijssens,M., Peeters,K., Rogaeva,E., De Jonghe,P., Cras,P., Martin,J-J., De Deyn,P.P., Van Broeckhoven,C., **Cruts,M.**, BELNEU consortium: The C9orf72 repeat size correlates with onset age of disease, DNA methylation andtranscriptional downregulation of the promoter. Molecular Psychiatry (2015) Epub: 20-Oct-2015 (PMID: 26481318) (I.F.: 14.496)
- **Gijselinck,I.**, Van Mossevelde,S., van der Zee,J., Sieben,A., Philtjens,S., Heeman,B., Engelborghs,S., Vandenbulcke,M., De Baets,G., Bäumer,V., Cuijt,I., Van den Broeck,M., Mattheijssens,M., Peeters,K., Rousseau,F., Vandenberghe,R., De Jonghe,P., Cras,P., De Deyn,P.P., Martin,J-J., **Cruts,M.**, Van Broeckhoven,C.: Loss of TBK1 is a frequent cause of frontotemporal dementia in a Belgian cohort. Neurology (2015) Epub: 18-Nov-2015 (PMID: 26581300) (I.F.: 8.268)
- Kleinberger,G., Capell,A., Brouwers,N., Fellerer,K., Sleegers,K., **Cruts,M.**, Van Broeckhoven,C., Haass,C.: Reduced secretion and altered proteolytic processing caused by missense mutations in progranulin. Neurobiology of Aging (In press) (I.F.: 5.013)

6.2. Articles in Books

- **Cruts,M.**, Engelborghs,S., van der Zee,J., Van Broeckhoven,C.: C9orf72-related amyotrophic lateral sclerosis and frontotemporal dementia, In: *GeneReviews®* [Internet] Edited by Edited by Roberta A Pagon, Editor-in-chief, Margaret P Adam, Holly H Ardinger, Thomas D Bird, Cynthia R Dolan, Chin-To Fong, Richard JH Smith, and Karen Stephens. (Seattle (WA): University of Washington, Seattle; 1993-2015; http://www.ncbi.nlm.nih.gov/books/NBK268647/): (2015)
- Cruts,M., Van Broeckhoven,C.: Genetics of frontotemporal dementia and related disorders, In: *Hodges' Frontotemporal Dementia* second edition Edited by Bradford C.Dickerson (Cambridge University Press) (In Press)

6.3. Abstracts in International Journals

- **Gijselinck,I.**, Van Mossevelde,S., Sieben,A., Heeman,B., Engelborghs,S., Vandenbulcke,M., Cuijt,I., Van den Broeck,M., Peeters,K., Mattheijssens,M., Vandenberghe,R., De Jonghe,P., Cras,P., De Deyn,P.P., Martin,J-J., **Cruts,M.**, Van Broeckhoven,C.: Loss-of-Function mutations in TBK1 are frequently associated with frontotemporal lobar degeneration in a Belgian patient cohort. Alzheimer's and Dementia (In Press)

6.4. Abstracts in Abstract Books of International Meetings

- Van Broeckhoven,C., **Gijselinck,I.**, Van Mossevelde,S., van der Zee,J., Sieben,A., Heeman,B., Engelborghs,S., Vandenbulcke,M., Vandenberghe,R., De Jonghe,P., Cras,P., De Deyn,P.P., Martin,J-J., **Cruts,M**., BELNEU consortium, EU EOD consortium: Loss-of-function mutations in TBK1 are a frequent cause of frontotemporal dementia and amyotrophic lateral sclerosis in Belgian and European cohorts. American Society of Human Genetics 2015, Baltimore, MD, USA, October 6-10 : 105 (Oral) (2015)
- **Cruts,M.**, **Gijselinck,I.,** Van Mossevelde,S., van der Zee,J., Sieben,A., Engelborghs,S., De Bleecker,J., Ivanoiu,A., Deryck,O., Edbauer,D., Zhang,M., Heeman,B., Rogaeva,E., De Jonghe,P., Cras,P., Martin,J-J., De Deyn,P.P., Van Broeckhoven,C., BELNEU consortium: The C9orf72 repeat expansion modulates onset age of FTD-ALS through increased DNA mathylation and transcriptional downregulation American Society of Human Genetics 2015, Baltimore, MD, USA, October 6-10 : 106 (Oral) (2015)
- Wauters,E., **Gijselinck,I.**, Van Mossevelde,S., Sieben,A., Van Langenhove,T., Mattheijssens,M., Peeters,K., Martin,J-J., van der Zee,J., Sleegers,K., Van Broeckhoven,C., **Cruts,M.**: An integrative approach to identify onset age modifier genes in an extended Belgion GRN founder family EMBO/EMBL Symposium: Mechanisms of neurodegeneration, Heidelberg, Germany, June 14-17 : 253 (Poster) (2015)
- Philtjens,S., **Gijselinck,I.**, Van Mossevelde,S., Sieben,A., Martin,J-J., van der Zee,J., Van Broeckhoven,C., **Cruts,M.**: Novel missense mutations in the vacuolar protein sorting 13 homolog C gene are associated with decreased endogenous protein expression in frontotemporal lobar degeneration. EMBO/EMBL Symposium: Mechanisms of neurodegeneration, Heidelberg, Germany, June 14-17 : 192 (Poster) (2015)
- **Gijselinck,I.**, Van Mossevelde,S., Sieben,A., Heeman,B., Engelborghs,S., Vandenbulcke,M., Cuijt,I., Van den Broeck,M., Peeters,K., Mattheijssens,M., Vandenberghe,R., De Jonghe,P., Cras,P., De Deyn,P.P., Martin,J-J., **Cruts,M**., Van Broeckhoven,C.: Loss-of-Function mutations in TBK1 are frequently associated with frontotemporal lobar degeneration in a Belgian patient cohort. Alzheimer's Association International Conference 2015 (AAIC 2015), Washington, USA, July 18-23 : DT-02-01 (Oral) (2015)

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 2015

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.

The experiments performed in 2014 regarding the first two aims resulted in several poster and oral presentations and the publication of a research paper in Neurobiology of Disease (Impact Factor 2014= 5.078). We have made significant progress regarding the remaining aims, resulting in a research paper that will be submitted in the first trimester of 2016. In addition, we have initiated another study based on aims A3 and A4.

1.2. (A1&2) Brain inflammation in a chronic epilepsy model: Evolving pattern of the translocator protein during epileptogenesis. (Amhaoul et al., Neurobiol Dis 2015)

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).

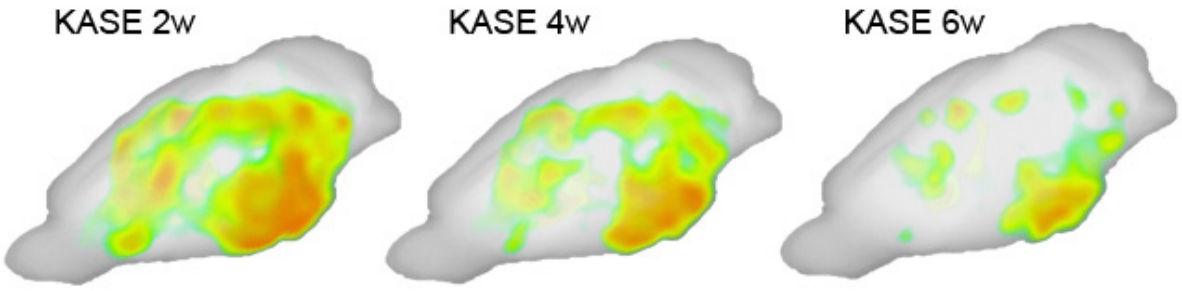

Figure 1: Serial 3-D ^{18}F-PBR111 PET images of a representative kainic acid-induced *status epilepticus* (KASE) rat merged with its MRI showing brain areas with increased brain inflammation. TSPO binding is peaking around 2 weeks post *status epilepticus,* a turning point when the first spontaneous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

1.3. (A3) the effect of TSPO on brain inflammation and steroid synthesis

To start investigating the link between TSPO and steroid synthesis, we have analysed several brain samples for the presence of neurosteroids allopregnanolone and pregnenolone sulphate with chromatography together with our collaborator Prof. Giuseppe Biagini (Unimore, Italy). Due technical difficulties in the sample analysis we have no established new connections with Prof. Michael Schumacher (Université de Paris Sud) to continue these efforts.

1.4. (A4) TSPO PET imaging as a biomarker to predict seizure and behavioural outcome (to be submitted)

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 the 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. We characterized the evolution profile of TSPO at 2 and 4 weeks post *status epilepticus* during epileptogenesis. Animals were recorded by video-EEG monitoring during the experiment. The recording started the day of the induction of *status epilepticus* for the entire period of the study (3 months) in order to obtain an exhaustive profile of the seizures (e.g. latency first seizure, seizures severity, seizure burden, and seizures frequency) for each animal. In addition, several behavioural test indicative of anxious and depressive behaviour, were performed.
The comparison of TSPO PET scans using network analysis and principal component analysis (PCA) showed difference among KASE animals that developed a high number of seizures and those who developed few seizures and controls (Figure 2). Moreover, we performed partial least square regression based on TSPO PET scans. This method resulted in a reliable model to predict the number of seizures that the animals will experience. In addition, this study confirmed the previous finding that KASE animals with few seizures have a higher expression of TSPO than KASE animals with seizures.

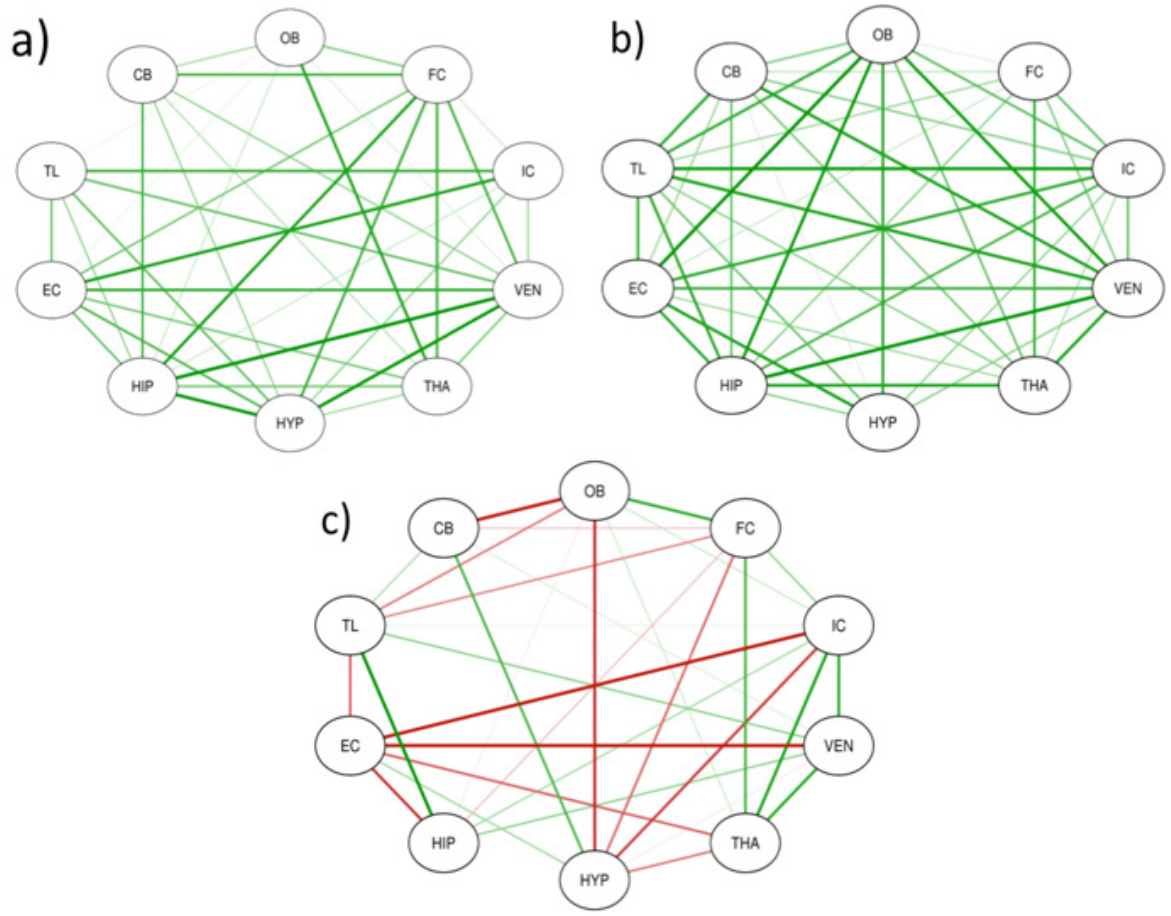

Figure 2. Network representations of neuroinflammation 2 weeks after *status epilepticus*. a) Controls-based network (n = 6). The most of the regions show positive correlations between each other suggesting an evenly distributed activity. b) KASE few seizures-based network (n = 7). Similarly to the controls, all the regions are positively correlated to each other. However, TSPO expression is significantly higher compared to the controls. c) KASE high number of seizures-based network (n = 5). Although TSPO expression is significantly higher than the controls, the network appears disrupted and the different regions do not equally express TSPO. Green edges represent positive correlations, red edges negative correlations. Nodes represents brain regions: OB = olfactory bub, FC = frontal cortex, IC = insular cortex, VEN = ventricles, THA = thalamus, HYP = hypothalamus, EC = endopiriform cortex, TL = temporal lobe, CB = cerebellum, WB = whole brain.

In addition, TSPO PET scans during epileptogenesis showed a clear relationship with behavioral tests performed during the chronic phase to determine co-morbidities (Figure 3). Whiskers nuisance task (WNT) has been performed 9 weeks post-SE (Figure 3a and 3b). This test reflects damages and circuitry alteration of the somatosensory cortex, taking into account anxiety and aggressiveness of the animals. KASE rats showed significant higher scores than controls ($p<0.01$), moreover the results positively correlated with TSPO levels during epileptogenesis in several brain regions (hippocampus, temporal lobe, insular cortex, endopiriform cortex, ventricles, and hypothalamus). Depression-like behavior has been tested 12 weeks post-SE by means of sugar preference test (SPT) (Figure 3c and 3d). Sugar preference during the chronic phase was significantly lower in KASE rats ($p<0.01$), and it negatively correlated with TSPO levels during epileptogenesis in several brain regions (hippocampus, temporal lobe, insular cortex, ventricles).

The approaches presented in this study could be useful in identifying subjects who will develop epilepsy in the future. In addition, these results suggest the possible role of TSPO PET imaging as a biomarker to predict the future development of comorbidities before the first seizure occurs. Altogether, these findings propose TSPO as an important player during epileptogenesis in the progress of epilepsy and its co-morbidities and could guide rational interventions studies.

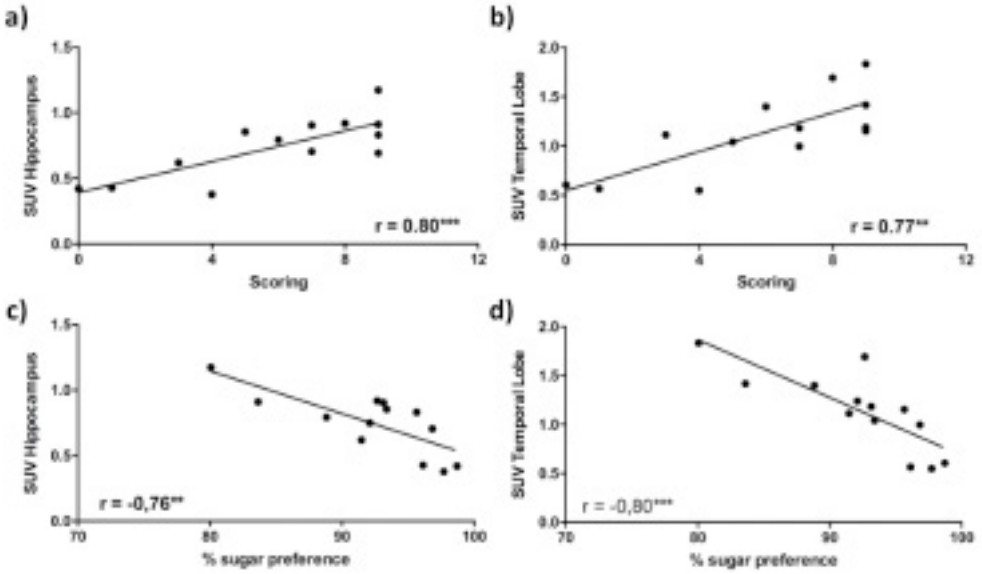

Figure 3. Relationship between TSPO during epileptogenesis and co-morbidities during the chronic phase. A positive and significant relationship can be established between TSPO expression in hippocampus 2 weeks post-SE and WNT scores 9 weeks post-SE (a) as well as with the temporal lobe (b). A negative and significant relationship can be established between TSPO expression in hippocampus 2 weeks post-SE and SPT 12 weeks post-SE (c) as well as with the temporal lobe (d). Pearson correlation test. **p<0.01,***p<0.001.

2. New opportunities

Since KASE animals with few seizures have a higher expression of TSPO than KASE animals with more seizures, we will verify the possible protective function of TSPO in limiting seizure burden. Just very recently, we agreed with our collaborator Prof. Marie-Claude Grégoire and Prof. Richard Banati from ANSTO to use a recently generated TSPO KO mice line. This study will allow us to discern the effect of TSPO on brain inflammation and to evaluate the role of TSPO during disease ontogenesis of epilepsy (A4). This is planned for the final year.

Based on the findings we obtain from the studies described above (A4 and TSPO KO mice), we will perform a pharmacological intervention targeting neuroinflammation or TSPO to further clarify its (patho)physiological role during epileptogenesis. We will treat animals either with an anti-inflammatory compound (P2X7 receptor antagonist), TSPO agonist or antagonist and evaluate the seizure outcome by video-EEG monitoring. TSPO expression will be investigated by means of TSPO PET imaging, and measurements of TSPO downstream pathways will be performed (e.g. neurosteroids and ATP) as well as histological assessment of neuroinflammation. This study will enable us to investigate the potential of TSPO modulation as a disease-modifying treatment and clarify the role of TSPO in pathogenesis.

3. Budget

As outlined in the description of the project proposal and last year's report, the 2015 budget received from the GSKE was amended for the salary of the laboratory assistant, to support animal purchase, various lab consumables and to present our results at the highly recognised international WONOEP meeting (see budget overview 1 & 2). The next year, the remaining funding will be required to cover the salary of an academic employee and some consumables, which is mandatory to sustain our research. Additional expenditures will need to be complemented by internal funding.

4. Scientific activities

4.1. Publications acknowledging G.S.K.E support in chronological order:

1. 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.
2. **Amhaoul H**, Staelens S, **Dedeurwaerdere S**. Imaging brain inflammation in epilepsy. Neuroscience. 2014 Oct 24;279:238-52.
3. **Amhaoul H,** Hamaide J, **Bertoglio D,** 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: Evolving pattern of the translocator protein during epileptogenesis. Neurobiol Dis. 2015 Oct;82:526-39.

3.1. Presentations at national and international scientific meetings

Invited Lectures

Stefanie Dedeurwaerdere

10/2/2015	**Speakers' series of the German Center for Neurodegenerative Diseases (DZNE):** *Nuclear imaging of maladaptive repair in acquired epilepsy models: opportunities for translational investigations of neuroinflammation and extracellular matrix alterations*	Magdeburg, Germany
2/11/2015	**1st ECMED workshop,** *Animal models of epilepsy*	Magdeburg, Germany
30/09/2015	**UCB summit I,** *Imaging of brain inflammation biomarkers in epilepsy*	Braine l'Alleud, Belgium

Oral presentation:

Stefanie Dedeurwaerdere

18-20/03/2015	**European Molecular Imaging Meeting (EMIM) 2015:** *Identifying brain network dysfunctions during epileptogenesis in a model of temporal lobe epilepsy*	Tubingen, Germany
03/09/2015	**WONOEP 2015:** *Identifying brain network dysfunctions during epileptogenesis in a model of temporal lobe epilepsy*	Istanbul, Turkey

Halima Amhaoul

01-06/02/2015	**Hot Topics in Molecular Imaging (TOPIM) 2015:** *Translocator protein as a neuroimaging biomarker of epileptogenesis in a rodent model of temporal lobe epilepsy.*	Les Houches, France

Poster presentations (presenting author)

Halima Amhaoul

18-20/03/2015	**European Molecular Imaging Meeting (EMIM) 2015:** *Characterising the spatiotemporal evolution of the translocator protein using post mortem and in vivo techniques in the KASE model of temporal lobe epilepsy.*	Tubingen, Germany

Daniele Bertoglio

11/03/2015	Sectie Wetenschappelijk Onderzoek (SWO) Meeting 2015: *Identifying brain network dysfunction during epileptogenesis in a model of temporal lobe epilepsy.*	Amsterdam, The Netherlands
22/05/2015	11th Meeting of the Belgian Society for Neuroscience: Identifying brain network dysfunction during epileptogenesis in a model of temporal lobe epilepsy.	Mons, Belgium

Geneeskundige Stichting Koningin Elisabeth
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Ongoing collaborations:

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

1. Brief clinical introduction

Patients with *gelsolin amyloidosis* (Familial Amyloidosis of the Finnish type) diplay diverse clinical symptoms including early aging, periferal neuropathies affecting the cranial nerves, bilateral progressive facial paralysis, and corneal lattice dystrophy. Recent neuropsychological tests showed abnormalities in visuocontructional and -spatial performance in patients (Kantanen et al., 2014). 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 take the first step in countering this disease using nanobody technology. Our strategy is in principle applicable to other neurological disorders.

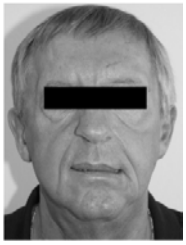

Figure 1. Bilateral upper and lower facial palsy in a FAF patient, caused by amyloid deposition in facial nerves. Reproduced from Luttmann et al., 2010.

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 novel mutation, encoding a N211K protein variant (asparagine to lysine mutation at position 211 in the primary structure) was reported (Efebera et al., 2014) and is associated with proteinurea as the only presenting symptom. A **mouse model** recapitulating the affliction (Page et al., 2009) is available in our lab. In this model, mutant gelsolin is engineered to be secreted from muscle fibers. Hence, we study the pathology by analyzing muscle contractility.

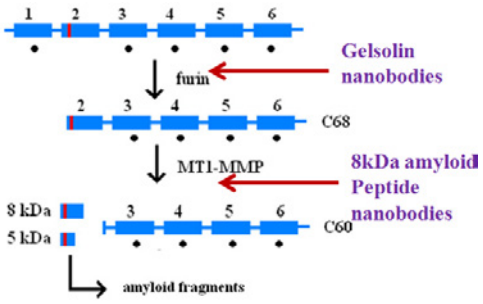

Figure 2. Mutant gelsolin domain 2 (red vertical line) is unable to bind calcium (black circle) and is cleaved twice during secretion, by furin and MT1-MMP, resulting in 8 kDa & 5 kDa peptides.

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., 2013a,b; Van Impe et al., 2008, 2013; Van den Abbeele et al., 2010; Bethuyne et al., 2014; Van Overbeke et al., 2014, 2015; Van Audenhove et al., 2013-2016). 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 amyloidogenic peptide.

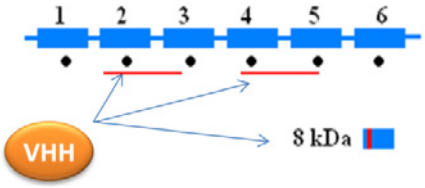

Figure 3. Schematic, showing where in gelsolin the 3 sets of nanobodies bind. The horizontal red lines indicate the epitope determined by pull down assays using GST fusions of gelsolin fragments and nanobodies, by approximation (Van den Abbeele et al., 2010).

3.1. First approach. Curtailing formation of amyloidogenic peptides through indirect blocking of MT1-MMP.

We present a brief summary here because this study is published (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 identified nanobodies (raised against the gelsolin 8 kDa peptide) that protect gelsolin against degradation by MT1-MMP. We termed them FAF nanobodies 1-3 and they act as a molecular chaperone by shielding gelsolin against MT1-MMP.

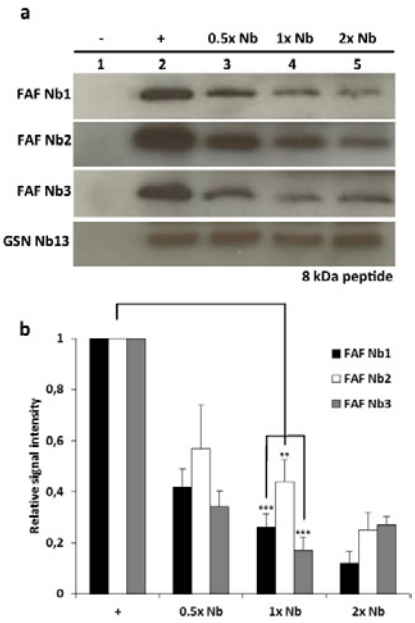

Figure 4 (a) FAF Nb1-3 were incubated with C68 (molar ratio nanobody:C68 indicated on top) prior to addition of MT1-MMP. The 8 kDa peptide was detected with anti-His HRP coupled antibody. Lane 1; negative control without MT1-MMP; lane 2: positive control with MT1-MMP but without addition of a nanobody. 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**.

We next showed that these nanobodies *interact with their target after intraperitoneal injection* in the diseased animals where gelsolin is degraded by furin and MT1-MMP (Figure 5).

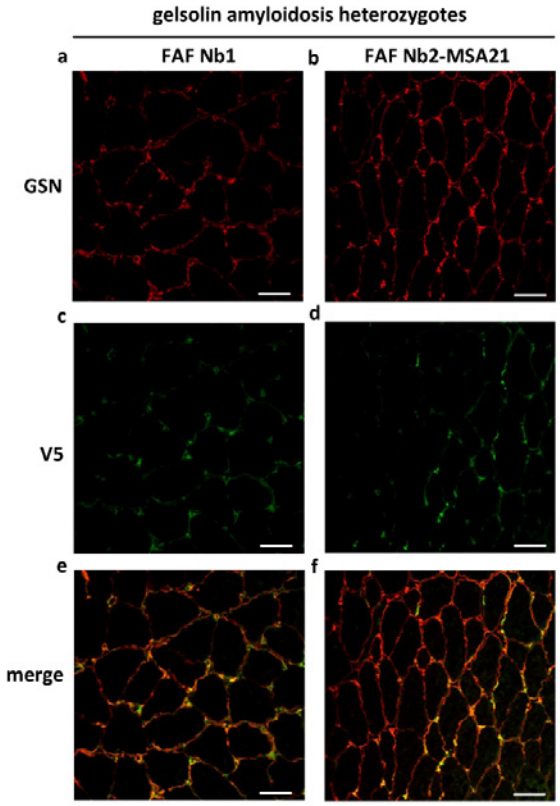

Figure 5: Intraperitoneally injected FAF Nb1 and FAF Nb2-MSA21 co-localize with gelsolin deposits in the endomysium of heterozygous gelsolin amyloidosis mice. Confocal microscopy images of heterozygous 5.5 month old gelsolin amyloidosis mice injected with 100 µg V5-tagged FAF Nb1 or FAF Nb2-MSA21. Musculus 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 injected 100 µg of recombinant nanobody weekly. A control group of heterozygous mice was injected with 200 µL PBS at the same time intervals. Typical features of contractile fatigue in 2 different hind leg muscles (extensor digitorum longus (EDL) and soleus), such as the decrease in relaxation rate were attenuated in nanobody-injected mice, compared to PBS. Hence we conclude that nanobodies reduce amyloid deposits around muscle tissue and improve its contractile properties (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).

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

Using this strategy we aimed to prevent the first cleavage step of gelsolin. 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 this nanobody might interfere with furin proteolysis, which turned to be indeed the case (Figure 6).

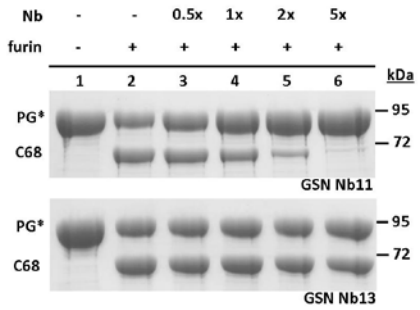

Figure 6. *In vitro* furin cleavage assay in which addition of furin to mutant plasma gelsolin (PG*) generates C68. Pre-incubation of GSN Nb11 (upper panel) with PG* reduced the amount of C68 generated by furin proteolysis in a concentration dependent manner. Negative and positive controls were included (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 (a negative control nanobody), irrelevant to furin proteolysis, had no reducing effect on C68 formation (lanes 3-6).

We next reared transgenic mice (the first mice that express a nanobody) that secretes gelsolin Nb11/13 for subsequent cross breeding with the gelsolin amyloidosis mice. These findings are published in the second paper (Van Overbeke et al., 2015). Briefly, GSN amyloidosis/nanobody double positive mice will express mutant gelsolin as well as nanobody where the latter protects degradation of the former. We examined muscle contractile properties and found that the decrease in contraction speed was strongly attenuated, across the entire 8-minutes fatigue protocol, in EDL muscles, but not in soleus of the GSN Nb11 mice compared to littermate controls (Fig. 7a,b). Hence, as in the previous approach, we can improve physiological muscle performance of these lab animals using nanobody technology, again attesting to the therapeutic potential of gelsolin nanobodies.

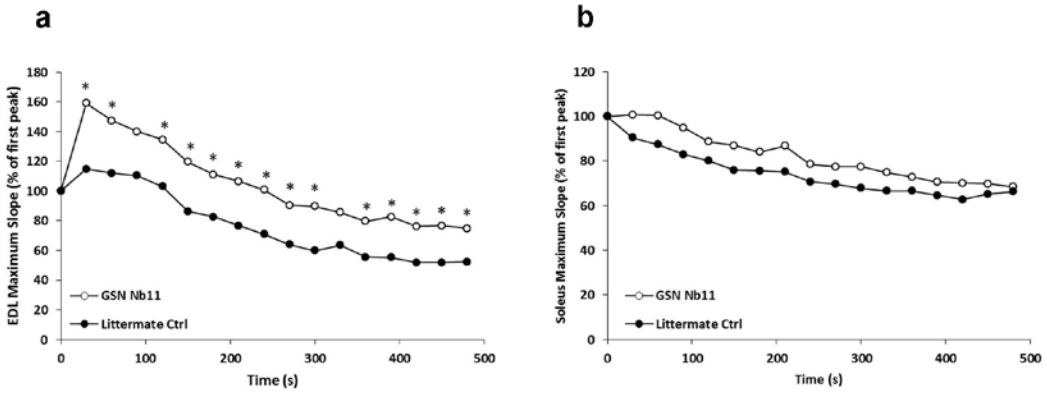

Figure 7. **Muscle contractile properties of gelsolin Nb11 expressing heterozygotes and their littermate controls.** Repeated *in vitro* muscle contractions in GSN Nb11 gelsolin amyloidosis mice (white dots) compared to littermate controls (black dots). (a,b) GSN Nb11 expression resulted in an attenuation 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 published in *Human Molecular Genetics* (Van Overbeke et al., 2015).

3.3. Visualization of amyloid fibrils in diseased animals using ^{99m}Tc-labeled nanobodies.

A third important goal in this project was to study disease progression in the same animal over time in a non-invasive manner. 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. Drs. Verhelle from our lab has completed this study. He visited the Lahoutte lab in Brussels (with whom we collaborate) on several occasions and has also attended a three day training course in Brussels to become acquainted with SPECT/CT imaging.

As a proof of concept, we implemented a ^{99m}Tc-nanobody in a follow-up study of the gelsolin Nb11-expressing mouse model (the same animals as descrbied in the previous section). Using biodistribution analysis and immunohistochemistry we demonstrated the validity of the data acquired via 99mTc-FAF Nb1 SPECT/CT. Our findings demonstrate the potential of nanobodies as a non-invasive tool to image amyloid deposition (Figure 8). We think that this approach can be extended to other amyloid diseases.

This study has also been completed and is currently under review in the *European Journal for Nuclear Imaging (third paper).* Furthermore, our first two papers have attracted attention. Indeed, we have been invited to contribute a chapter to a new book, under editorship of Dr. Ana Maria Fernandez-Escamilla, entitled “Amyloids”. By March 8, 2016 we will submit a chapter carrying the title A nanobody based approach to amyloid diseases. The gelsolin case study, by Verhelle, A., Van Overbeke, W. and Gettemans, J.

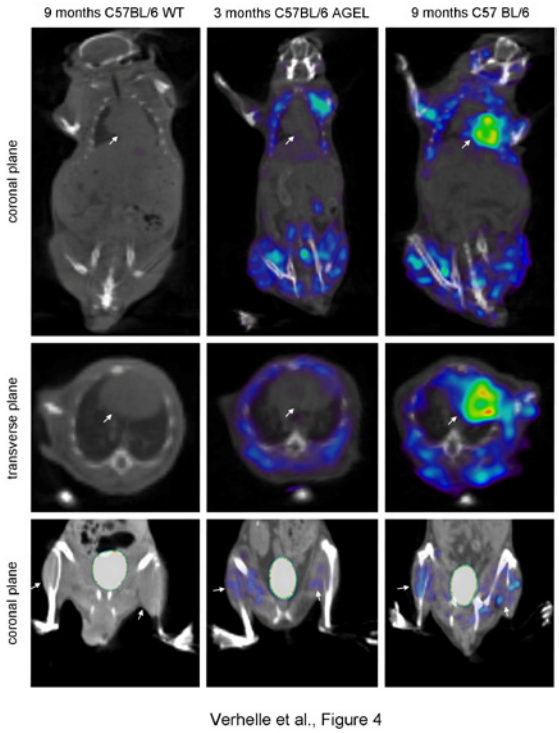

Figure 8. SPECT/CT images obtained with ^{99m}Tc-FAF Nb1. In WT mice (left) no significant background signal can be detected. In AGel mice from 3 months onwards (middle and right), the major muscle groups clearly light up. The heart however, seems unaffected at 3 months (middle) but gives a high signal at 9 months (right). White arrows indicate the heart (upper 2 panels) and major muscle groups of the hind legs (lower panel).

3.4. Simultaneous protection of gelsolin against furin *and* MT1-MMP.

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 collaboration with **prof. Marinee Chuah and prof. Thierry Vandendriessche (**Department of Gene Therapy & Regenerative Medicine, Free University Brussels) we generated adeno associated viral particles (AAV) with a tandem nanobody cloned into their genome (Figure 9), because they represent an efficient tool to obtain high level and long term transduction and expression in vivo. The tandem nanobody consists of Nb11 that protects against furin degradation whereas the second nanobody protects against MT1-MMP. Furthermore the sequence that connects both nanobodies is a kind of decoy because it is a consensus sequence for MT1-MMP.

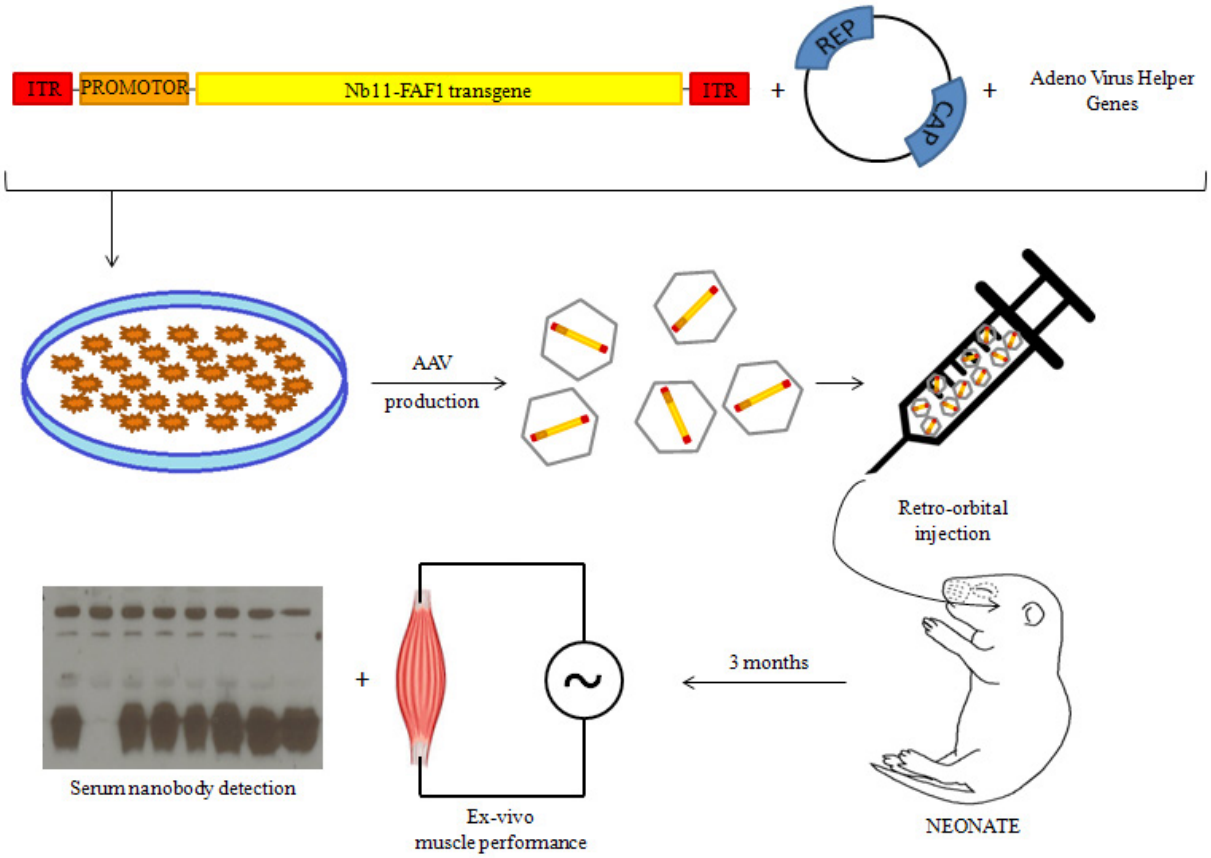

Figure 9. Flowchart of the methodology used to prevent gelsolin degradation in diseased mice using adeno associated virus particles (AAV) expressing a tandem nanobody construct. Analysis of the data is currently ongoing.

Other noteworthy events

* On Friday the 16th of January 2015, Drs. Van Overbeke defended his PhD work in public, entitled: **Keeping mutant plasma gelsolin safe from harm: gelsolin nanobodies act as a chaperone against pathological proteolysis.** He is currently employed as a post-doc at the University of Malmo in Sweden.

* Adriaan Verhelle will defend his PhD work at the end of this year or the beginning of next year.

4. Summary

4.1. Papers resulting from this study:

1. 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.
2. 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. 2015. *Human Molecular Genetics.* 24, 2492-507.
3. A nanobody based approach to amyloid diseases. The gelsolin case study, by Verhelle, A., Van Overbeke, W. and Gettemans, J. Book chapter, Dr. Ana Maria Fernandez-Escamilla (editor), "Amyloids". In preparation.
4. Verhelle, A., Van Overbeke, W., Peleman, C., De Smet, R., Zwaenepoel, O., Lahoutte, T., Van Dorpe, J., Devoogdt, N., Gettemans, J. Non-invasive imaging of amyloid deposits in a mouse model of AGel using ^{99m}Tc-modified nanobodies and SPECT/CT. Under review.
5. Verhelle, A. et al. Paper describing the results obtained with AAV virus in transgenic mice. To be submitted in the course of 2016. This paper is required to allow A. Verhelle to obtain his PhD degree.

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Regulation of waking brain function by sleep debt, circadian and seasonal rhythms

1. Introduction

During this year, we finalized a number of analyses and submitted several manuscripts dealing with the regulation of human sleep and wakefulness**.**

2. Regional modulation of human brain responses by circadian rhythmicity and time awake

At any point in time, human performance results from an interaction between circadian rhythmicity and sleep pressure accumulated during wakefulness. How this interaction is reflected in local brain responses is not known. Here we quantify changes in brain responses to two cognitive tasks during functional magnetic resonance imaging (fMRI) sessions scheduled across the circadian cycle during 42h of wakefulness. Results demonstrate that the temporal profile of brain responses reflects the combined influence of sleep pressure and circadian rhythmicity, and that their contribution varies across brain regions. Prefrontal responses are more strongly sleep-pressure related, while subcortical areas are under a prominent circadian modulation, apparently contributing to preserved performance during the biological day despite accumulated sleep pressure. This subcortical/prefrontal “dichotomy” was maintained across responses to the attentional and working memory tasks. These findings have implications for the understanding of the brain mechanism involved in deterioration of cognition such as observed in shift work and ageing.

This paper is submitted for publication.

3. Seasonality in human cognitive brain responses

Seasons correspond to annual environmental fluctuations to which organisms have adapted. Yet, little is known about seasonal variations in human brain physiology. We investigated annual rhythms of brain activity in a cross-sectional study of healthy young participants. They were maintained in a season cue-free environment for 4.5 days after which brain response were assessed using functional magnetic resonance imaging (fMRI) while performing two different cognitive tasks. Brain responses to both tasks varied significantly across seasons, but the phase of their annual rhythms was strikingly different. For the sustained attention task, the maximum and minimum responses were located around the summer and winter solstices, respectively, while for the working memory task, maximum and minimum responses were observed around the autumn and spring equinoxes. These findings reveal previously unappreciated process-specific seasonality in human cognitive brain function which may be related to photoperiod and could contribute to intra-individual cognitive changes at specific times-of-year.

Daily variations in the environment have shaped life on Earth, with circadian cycles identified in most (if not all) living organisms. Seasonal variations in the environment have triggered annual adaptations which are observed in the majority of living organisms. Yet, limited seasonal variations have been reported in our species.

Mood is the only aspect of human brain function which is truly accepted to be affected by season, with suicidality and symptoms of several mood disorder undergoing marked seasonal variations. A large portion of the population undergoes seasonal mood variations which do not reach clinical symptoms [e.g. subsyndromal seasonal affective disorder (SAD): up to 18% in North America]. Some studies also suggest seasonal changes in cognitive brain function in the general population, but results are not consistent. Seasonal change in photoperiod is very likely to play a role in these annual variations, but the mechanisms involved remain debated. Although neurotransmitters and neurotrophic factors potentially mediating seasonal mood variation have been identified, the brain bases of seasonality in cognition remain elusive.

Genuine seasonal rhythms of human brain function are difficult to measure because a number of factors which could directly affect brain function have to be controlled: light exposure, sleep/wake rhythm, external temperature, food intake, physical exercise, social interactions. We took advantage of a study completed in our laboratory to assess annual rhythms in human cognitive brain function. This cross-sectional study, was conducted in Liège (Belgium, latitude 50,633° N, longitude 5,567° E), between May 2010 and October 2011. Twenty-eight young healthy participants (age 21±1.5y.; 14F) were instructed to follow a regular sleep/wake schedule for 3 weeks prior to a 4.5 day in-lab protocol devoid of seasonal cues. Two fMRI recordings were acquired 1h after wake-up time following 63h under strictly controlled experimental conditions (Fig.1). Each recording included a task probing a different cognitive domain: a sustained attention task [visual psychomotor vigilance task, PVT], and a higher order executive functions tasks [auditory n-back task, involving storage, updating and comparison of information in working memory]. We hypothesized that brain responses to both tasks would undergo seasonal variations with higher and lower responses, respectively, around summer and winter solstices.

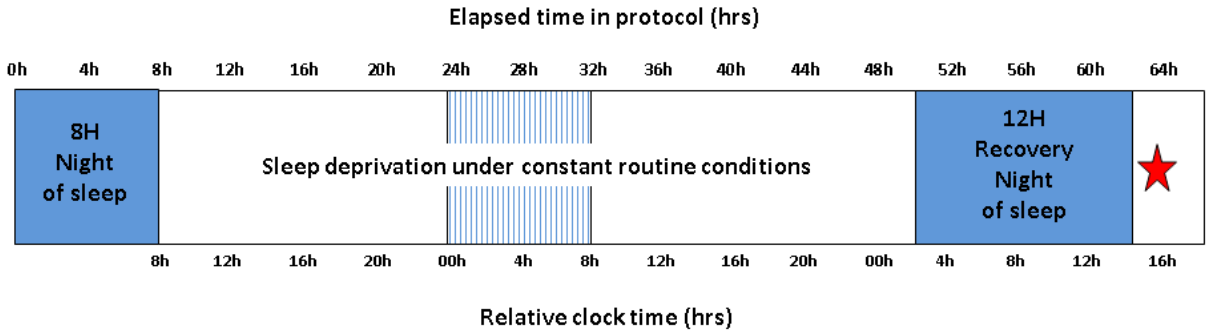

Fig. 1. Schematic representation of the strictly controlled part of the protocol. Following an 8h baseline night of sleep in complete darkness, participants underwent a 42h sleep deprivation under constant routine conditions in dim light (<5 lux, 19°C, semi-recumbent position, regular liquid isocaloric food intake, no time-cues, sound-proofed room). They were then given a 12h recovery sleep opportunity in darkness, an hour after which they completed fMRI. Functional MRI recordings were completed while laying down in darkness and included PVT and n-back task. Relative clock time for participants habitually waking-up at 8:00am.

We first focused on the brain responses induced by the PVT and found significant annual variations in areas involved in alertness [thalamus and amygdala], and in executive control[frontal areas and hippocampus] (Fig. 2A). Seasonal variations were also detected in the globus pallidus, parahippocampal gyrus, fusiform gyrus, supramarginal gyrus, and in the temporal pole recruited during PVT and in the precuneus involved in visuo-spatial attention. As postulated, extraction of the seasonal variations in PVT brain responses revealed a similar rhythm in all these brain regions, with maximal responses around mid-June, and minimal around mid-December, i.e. around solstices (Fig 2B).

Variations in PVT brain responses were not related to significant changes in PVT performance, which remained good and stable throughout the year ($p > 0.2$). This guarantees that fMRI differences were not biased by behavioral differences and suggests that fMRI is more sensitive than behavioral testing

in identifying seasonal variations in cognition. Stable performance throughout the year via distinct brain dynamics implies however that the "cost" of cognition, i.e. the neural resources involved in or at disposal for cognition, change with time-of-year. We hypothesize that the seasonality in brain responses could predict some of the seasonal variations in performance previously reported for potentially more sensitive tasks.

We next investigated whether other behavioral and physiological variables could account for the observed annual variations in PVT-brain responses. Subjective and objective neurophysiological measures of alertness and subjective assessments of affective dimensions acquired immediately before fMRI acquisitions, melatonin phase, midpoint, amplitude, duration of secretion during the preceding constant routine protocol and the timing of fMRI relative to melatonin phase did not change across season, as well as sleep characteristics during baseline and recovery did not show any significant seasonal variation in our protocol. Only self-reported mood varied significantly over season but this variation was not significantly related to the seasonal changes in brain responses. In summary, sustained attention-related brain activity fluctuates across seasons but these changes were not related to behavioral, endocrine or neurophysiological variations during sleep and waking.

Photoperiod is the most obvious factor associated with season and both the intensity and spectral composition of light to which people are exposed vary with season. Figure 2 indeed suggests that PVT brain responses were closely related to photoperiod (grey area, Fig.2B). A formal analysis revealed that all PVT brain responses showing seasonal variations were significantly associated with day length. This finding could imply that some 'physiological memory' for the photoperiod to which participants were exposed prior to admission to the laboratory, is present in humans. Indeed, participants at the time of the last fMRI session had not seen sunlight for 4.5 days and had been for 63h in dim-light during wakefulness and darkness during sleep episodes. Consistently, effects of prior light exposure ('photic memory') on cognitive brain responses have formerly been demonstrated on a much shorter timescale in human and photoperiod memory has previously been described as "after-effects" of photoperiod on circadian clock neurons. Whether our data reflect a true human photoperiod memory is, however, not possible to ascertain as many other environmental factors covary with season and photoperiod, including air temperature, and humidity (Figure 2E).

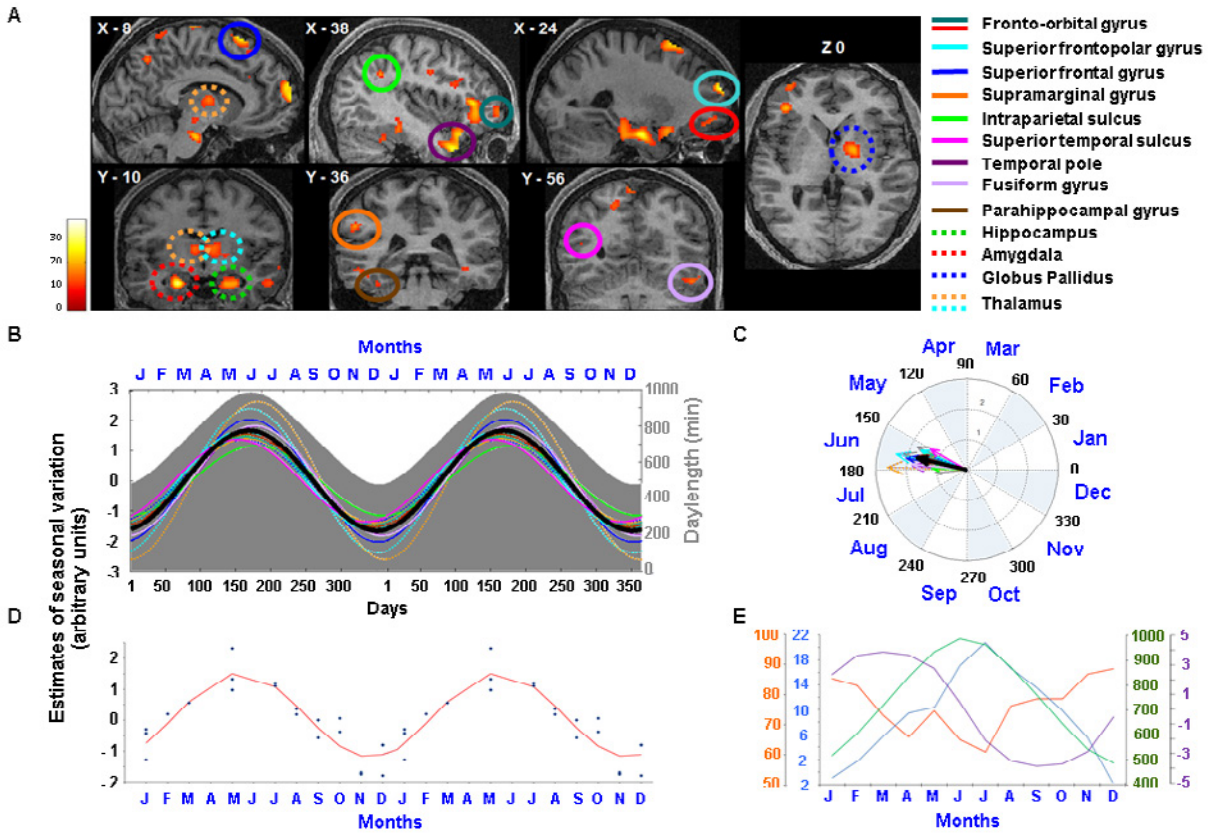

Fig. 2. Seasonal variations in brain activity associated with sustained attention. (**A**) Significant ($p_{corrected}$<.05) seasonal variations in PVT brain responses displayed over the mean structural image of all participants (display at $p_{uncorrected}$<.001). For clarity sake, only cluster >30 voxels are displayed (see Table S2 for full results). Vertical color bar corresponds to F-test values (**B**) Double plot of PVT-brain response estimates in regions of panel A in a sinusoidal representation. Day 1 corresponds to January 1st. First letter of each month is displayed on top. Thick black line corresponds to average of all response estimates. Grey area represents daily daylength (in minutes) in Liège. (**C**) Same as B in polar coordinates, arrow length represents seasonal variation amplitude. One degree is roughly equal to one day (360 degrees for 365 days). Maximum responses lay between 152 and 188 degrees (mean 168.9), i.e. June 3rd and July 9th (mean June 20th). (**D**) Double plot of individual activity estimates in a representative regions of A (amygdala) and its sinusoidal fit (**red** line). (**E**) Seasonal environmental factors: temperature (Celsius degrees; **blue**); humidity (%; **red**); daylength (min, **green**); day-to-day daylength gain/loss (min; **violet**).

Having established seasonal/annual variations in sustained-attention-related brain responses, we then examined whether such variations could be generalized to other cognitive domains by considering the n-back task implemented in our protocol. We found that executive activity varied significantly with season in the thalamus, including the pulvinar, and in prefrontal and frontopolar areas, similar to the PVT results. In addition, significant annual variation was observed in the insula, a brain regions involved in executive processes, attention and affective regulation. Compared with PVT brain responses, significant seasonal variation appeared to encompass a reduced set of brain areas, which could indicate a relative decrease in seasonality on executive brain responses, in line with previous suggestions of a reduced seasonal impact on behavioral measures of more complex task.

This qualitative task-specific difference was complemented by a statistically significant difference in the dynamics of brain response estimates across the year, with maximum and minimum responses being located approximately 3 months later for the n-back as compared to the PVT, i.e. around the autumn and spring equinoxes respectively (Fig. 3B-C) (day of the year at responses maximum phase: PVT: 168.9±8.2; n-back: 265.7±13; t_{11} = -20.16; $p < 0.001$).

Similar to the PVT, performance on the n-back was good and stable throughout the year in our sample. However, because of the annual rhythm phase delay, the co-variation with photoperiod was not significant for any of these executive brain responses that varied significantly with season. As depicted

on Figure 3, there seems, however, to be a striking similarity between annual dynamics in executive brain responses and day-to-day variation in daylength, i.e. the number of minutes of daylength gained or lost from one day to the next which peaks at the equinoxes. This similarity is indeed confirmed statistically. As for photoperiod, however, factors such as air temperature and humidity (Figure 2E) co-vary with day-to-day daylength variations such that these are equally likely to contribute to seasonality in cognitive brain function.

The results provide evidence for seasonality in diverse types of cognitive processes and suggest that the annual dynamics are process-specific. Causes of this specificity are unclear. One might postulate that more basic cognitive processes, such as attention, are more tightly related to basic environmental changes (e.g. daylength) while higher cognitive processes are more socially related (e.g. summer holidays encompass usually July and August in Belgium). This pure speculation cannot be tested here but would imply that brain response seasonal dynamics would be different in countries with different environmental and social constrains. Interestingly, seasonal variation in serum Brain-Derived Neurotrophic Factor (BDNF) concentration, a protein involved in learning and the regulation and plasticity of neuronal network, has been reported to undergo a similar annual dynamics than executive brain responses in our sample (*28*). Whether brain responses to learning tasks would have a similar annual pattern as the brain activity related to a working memory task remains to be investigated.

The influence of season is broad in animal kingdom and encompasses locomotion, body mass, endocrine function (melatonin secretion), fur production and sexual activity. In humans it was recently established that gene expression in white blood cells and adipose tissues varies significantly with season and we establish that seasonality includes higher cognitive brain functions. Our findings indicate that, in addition to time-of-day, time-of-year influence brain function in healthy participants. Our results have direct and important bearing on our understanding of intra-individual cognitive changes that could emerge at specific times-of-year. In a broader prospective, variations in cognitive brain function over the seasons may potentially explain changes in affective control in vulnerable populations, as in SAD.

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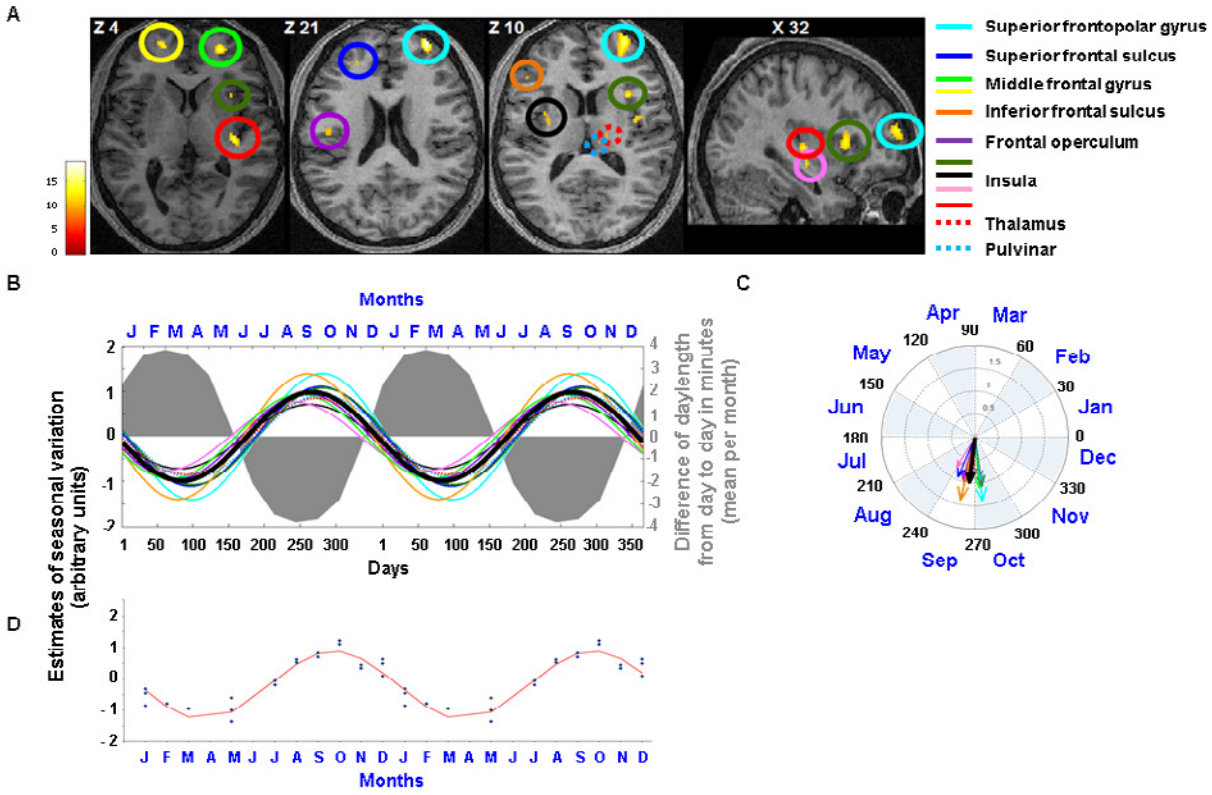

Fig. 3. Seasonal variations in executive brain activity. Display as in Figure 2. (**A**) Significant ($p_{corrected}$<.05) seasonal variations in auditory 3-back brain responses minus control task brain responses (simple letter detection). (**B**) Executive brain response estimates in regions of panel A. Grey area represents day-to-day change in photoperiod in Liege (minutes). (**C**) Same as B in polar coordinates. Maximum responses were located between 243 and 282 degrees (mean 265.75), i.e. September 3rd and October 12th (mean September 22th). (**D**) Double plot of individual activity estimates in a representative regions of A (middle frontal region) and its sinusoidal fit (**red** line).

4. Clock-dependent dynamics in human cortical excitability

Wakefulness extension increases cortical excitability, i.e. neuronal responsiveness, and is detrimental for cognitive performance. However, the circadian signal maintains cognition remarkably stable during a normal waking day before jeopardizing it if wakefulness is prolonged into the biological night. Whether the circadian timing system achieves this fine-tuned regulation through an impact on cortical excitability is unknown. Here, we assessed cortical excitability using scalp EEG recordings of direct (TMS) stimulations of the cortex in young individuals during a stringent sleep deprivation protocol. Data reveal a clock-dependent modulation of cortical excitability over the 24h day-night cycle. This non-linear modulation is strongest in those individuals with strongest circadian drive and is associated with macroscopic brain EEG and behavior changes. These results reveal cognition and system-level brain function is very likely to be shaped by direct circadian modulation of cortical excitability which could orient future pharmacotherapy and chronotherapy development.
These results are submitted for publication.

5. Human excitation/inhibition balance is under circadian influence

Neuronal activity during wakefulness is not stable across the 24h day-night cycle. Increasing evidence speaks for a clock-driven impact on neuronal function. However, how cortical dynamics are orchestrated by circadian processes remains an open question, particularly in humans. Here we show that human

excitatory/inhibitory balance in neuronal subpopulations of the prefrontal cortex fluctuates across time, through dynamics indicative of a strong circadian influence in addition to wake-duration dependent impact. Furthermore this non-linear dynamics was associated with fluctuation in performance and electroencephalogram (EEG) parameters. Our data provide a proof-of-principle for how the circadian timing system set basic neuronal function, and offer a unique window onto the hidden neuronal milieu that sets the pace for the temporal organization of human brain function.
These results are submitted for publication.

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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

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 reduced 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, has 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.

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]. 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].

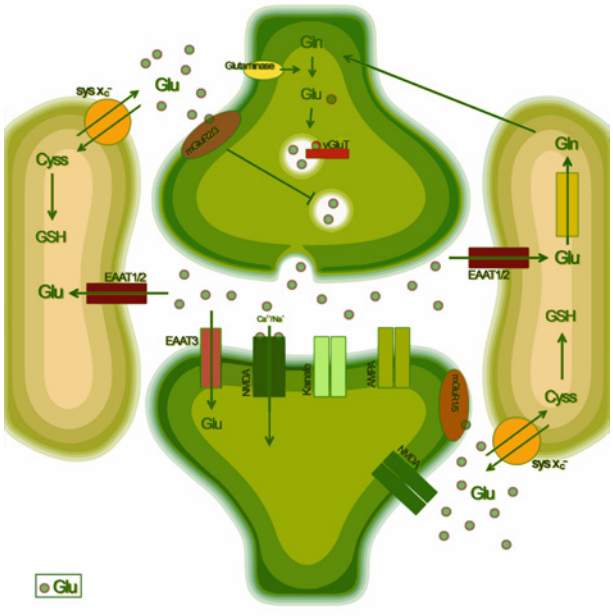

Figure 1: schematic representation of a glutamatergic synaps including glutamate reuptake transporters (EAAT), glutamate receptors (NMDA, AMPA, mGluR) and system x_c^- (sys x_c^-).

The **rationale for studying system x_c^- as a target for treating PD and its comorbidities** is based on the dual role this 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, investigate the effect of LAC- and MPTP-induced parkinsonism on the expression of xCT. **3/** 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, investigate the link between system x_c^- and anxiety/depression in healthy as well as parkinsonian mice. During the time-course of this project, **two additional studies**, related to this project, have been conducted in order to: **5**/ characterize antibodies directed against xCT and **6**/ investigate neuroprotective effects of zonisamide (ZNS).

Results obtained since the start of the project (2014-2015):
Results that were obtained in 2014 and already reported in our activity report of 2014 are in italic. For figures related to these results, we refer to our previous report.

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. 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 (fig. 2 in report 2014). In the near future, additional samples will be obtained from the Netherlands brain bank.

In this small number of samples we also investigated expression levels of proteins of the phosphoinositide 3-kinase (PI3K)/glycogen synthase kinase 3β (GSK-3β)/eukaryotic initiation factor 2α (eIF2α)/activating transcription factor (ATF4) pathway (collaboration with P. Maher, Salk Institute, San Diego, US and J. Lewerenz, University Hospital Ulm, Germany). We previously characterized this pathway as being responsible for the increased xCT expression levels in hippocampus of epileptic patients[22] as well as a chronic epilepsy mouse model (Leclercq, Van Liefferinge *et al.*, in preparation). Yet, in PD samples, there was no correlation between the changes in xCT expression and the proteins of this pathway. Further studies should identify the pathway that modulates xCT expression in PD.

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.

2.1. Characterization of the intranigral lactacystin mouse model

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 this **manuscript** *that was* **recently published**[23] *we report that unilateral administration of LAC to the SN of mice leads to acute and non-progressive dopaminergic neurodegeneration (fig. 3 in report 2014), in the presence of increased levels of Ser129-phosphorylated α-synuclein (fig. 4 in report 2014). These pathological changes induced the development of motor asymmetry and impairment, as assessed*

in various motor behavior paradigms (fig. 5 in report 2014). Furthermore, we detected signs of non-motor symptoms resembling early-stage Parkinson's disease, including somatosensory disturbances, akathisia (restlessness), perseverative behaviour (fig. 5 in report 2014), and anxiety-like behaviour (fig. 6 in report 2014). 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.

We are currently studying STN hyperactivity in this LAC model. Ongoing experiments should reveal whether the increased VGLUT2 protein expression (vesicular glutamate transporter) that we observe in the Substantia nigra pars reticulata (SNr) originates in the STN (*in situ* hybridization for VGLUT2) and whether the latter region is hyperactive (CO staining/zif268 *in situ* hybridization), conform observations in PD patients.

Finally, using electron microscopy (collaboration with C. Meshul; VA Medical Center, Portland, Oregon, USA) we are studying ultrastructural changes in corticostriatal synapses in the nigral LAC mouse model (fig. 2). Previous findings indicate significant synaptic plasticity in the striatum of patients with PD, characterized by an increase in the size of the post-synaptic densities of dendritic spines in asymmetric contact with afferents most likely of cortical origin[24]. In line with the proposed hyperactivity of cortico-striatal terminals in PD, we provide evidence suggesting an increase in the activity of the cortico-striatal pathway following intranigral LAC administration. In particular, by performing VGLUT1-pre-embed diaminobenzidine immunolabeling followed by post-embed immunogold on slices covering the dorsolateral striatum, we observed a significant increase in the percentage of labeled terminals containing mitochondria. As mitochondria are recruited into the presynaptic terminal with increased activity[25], this might indicate an increase in the activity of the corresponding corticostriatal afferents. Consistent with this notion, the post-synaptic elements contacted by the labeled terminals exhibited plastic changes suggestive of increased synaptic activity following lesion, including an increase in the thickness and area of the post-synaptic density.

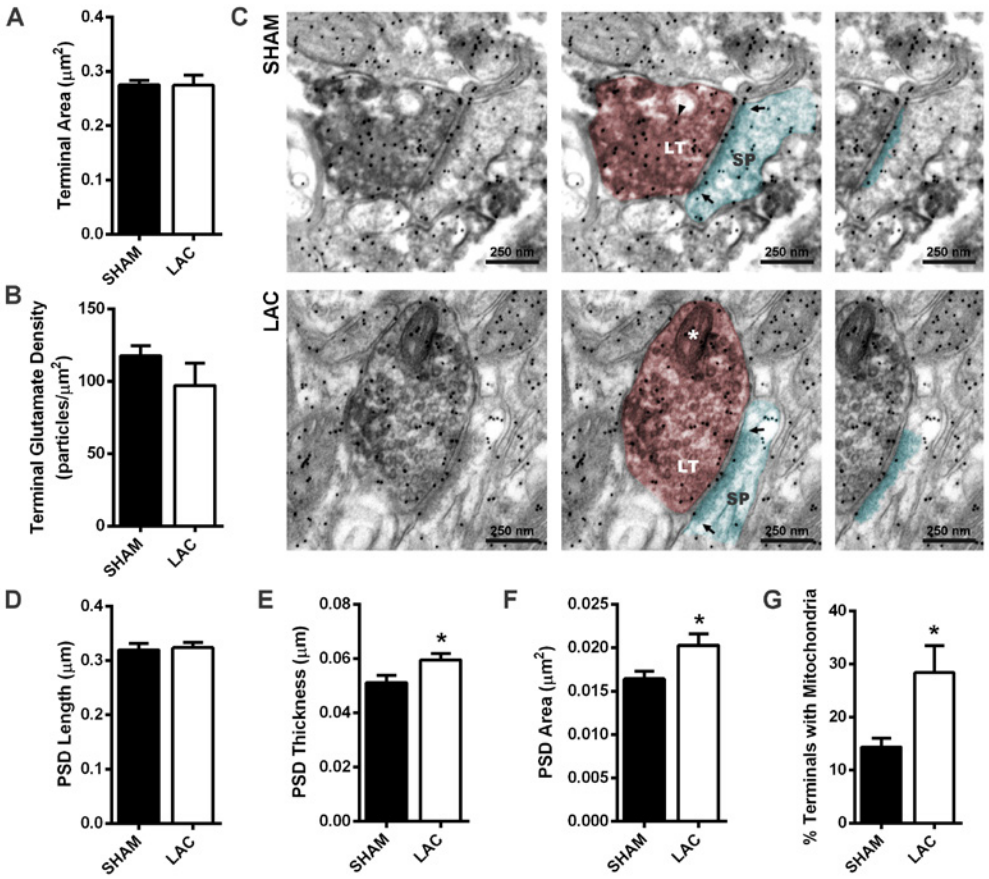

Figure 2: Plastic changes occurring at corticostriatal synapses in the lactacystin (LAC) mouse model of Parkinson's disease. Intranigral LAC injection led to significant modifications of corticostriatal synapses after 1 week, including increases in the thickness (**E**) and area (**F**) of the postsynaptic density (PSD) and percentage of corticostriatal terminals containing mitochondria (**G**). LAC administration did not influence the area (**A**) or density of glutamate (**B**) in corticostriatal terminals, nor did it change the length of the PSD (**D**). **C**: Representative electron micrographs of VGLUT1- pre-embed diaminobenzidine labeled terminals (LT, shaded red) contacting dendritic spines (SP, shaded teal) in the dorsolateral striatum of SHAM- (top; control mice) or LAC- (bottom) mice. The asymmetric axospinous contacts are defined with arrows. Glutamate molecules have been identified using post-embed glutamate immunogold (arrowhead). Note the increased presence of mitochondria in corticostriatal terminals of LAC-lesioned mice (asterisk). Right panel: The PSD has been highlighted (teal) in order to illustrate the increased thickness and total area following intranigral LAC injection. Data are presented as mean ± SEM and were generated from n=8 SHAM mice (22.5 ± 1.701 synapses/mouse) and n=7 LAC mice (24.86 ± 2.143 synapses/mouse). Statistical analysis was performed using an unpaired t test, *p<0.05.

2.2. Involvement of system x_c^- in lactacystin-induced parkinsonism

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 in report 2014). 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.

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 in report 2014) or behavioral impairment (fig. 9 in report 2014). 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 in report 2014).

The reason for this age-dependent protection is currently being elucidated as part of a larger study in which we investigate the role of system x_c^- in (healthy) aging.

2.3. Involvement of system x_c^- in MPTP-induced parkinsonism

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 C. Meshul and are **published in Neuroscience Letters[26].** *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.*

Our results indicate that the expression of xCT undergoes region-specific changes in MPTP-treated mice, with increased expression in the striatum (fig. 11A in report 2014), and decreased expression in the SN (fig. 11B in report 2014). 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 (fig. 12 in report 2014). 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.

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 will be performed in 2016.

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 recently **published in Progress in Neuropsychopharmacology & Biological Psychiatry[27].**

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 in report 2014). 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 in report 2014) and antidepressive-like (fig. 16 in report 2014) 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.

5. Specificity of antibodies raised against xCT

Many studies aiming to unveil the distribution or the role of system x_c^-, rely on antibody recognition of its specific subunit, xCT. It is generally accepted however that antibody specificity is a major problem. Until 2004, the only possibility to detect system x_c^- was the use of an antibody against a selective substrate, aminoadipic acid[28]. After that, distinct antibodies raised against xCT appeared without consensus about the molecular weight of the antiporter. Based on literature, the xCT protein band would be expected around 55.5 kDa[29]. However, it has been reported that our own antibody[17] as well as the antibodies raised by Burdo *et al.* (2006)[30], Shih *et al.* (2006)[31] and La Bella *et al*. (2007)[32] recognize a fuzzy protein band at about 35 kDa. Antibody specificity of our own polyclonal antibody was confirmed on brain tissue of $xCT^{-/-}$ mice[18], using Western blotting[17]. The presence of nonspecific protein bands (present in both $xCT^{-/-}$ and $xCT^{+/+}$ tissue) shows that this antibody also recognizes unrelated proteins and as such cannot be used for immunohistochemistry[17]. Many researchers are still identifying the 55.5 kDa sharp immunoreactive band, observed when using most commercial antibodies in Western blot experiments and claimed to be xCT on the datasheets accompanying these antibodies, as xCT. Since often these antibodies are even used for immunohistochemistry, wrong conclusions can easily be drawn. Moreover, inappropriate negative controls are frequently being used to control for antibody specificity, such as pre-adsorption of the antiserum against the peptide or omission of the secondary antibody[33,34].

We therefore verified the specificity of the most commonly used commercial as well as some in-house-developed anti-xCT antibodies (table 1, 2), by comparing their immunoreactivity in mouse brain tissue of $xCT^{+/+}$ and $xCT^{-/-}$ littermates using Western blotting (fig. 3) and immunohistochemistry (fig. 4, 5). A specific signal should be observed exclusively in $xCT^{+/+}$ tissue and be absent in $xCT^{-/-}$ tissue. The Western blot screening results demonstrate that antibody specificity not only differs between batches produced by immunizing different rabbits with the same antigen, but also between bleedings of the same rabbit (table 1). A number of immunohistochemical protocols has been tested for all the anti-xCT antibodies that were specific on Western blots (*in table 1 and 2) in order to obtain a specific immunolabeling (fig. 4). Only one of our in-house-developed antibodies could reveal specific xCT labeling and exclusively on acetone-postfixated cryo-sections (fig. 5). Using this approach, we observed xCT protein expression throughout the mouse forebrain, including cortex, striatum, hippocampus, midbrain, thalamus and amygdala, with highest expression in regions facing the cerebrospinal fluid and meninges (fig. 5). **The results of this study were published in the Journal of Comparative Neurology[35].**

Table 1: In-house developed antibodies that were tested in this study (* specific protein band detected).

Antibody ID	Production Date	Rabbit ID no	Peptide	Peptide sequence	Concentration (µg/ml)
Antibody#496	2002-10-27	1B1685	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.079
Antibody#497	2002-10-27	1B1685	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.028
Antibody#510	2004-07-14	1B1685	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.183
Antibody#511	2004-07-14	1B1685	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.292
Antibody#512	2004-07-14	1B1685	MxCT(492-502)	LEVVPEDSKEL-(COOH)	0.108
Antibody#418	2003-01-13	2B0438	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.092
Antibody#422	2003-01-13	2B0438	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.067
Antibody#502	2004-07-14	2B0635	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.042
Antibody#501	2004-07-14	2B0635	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.158
*Antibody#500	2004-07-14	2B0635	MxCT(492-502)	LEVVPEDSKEL-(COOH)	0.225
Antibody#504	2004-07-14	2B0636	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.117
Antibody#505	2004-07-14	2B0636	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.058
Antibody#506	2004-07-14	2B0636	MxCT(492-502)	LEVVPEDSKEL-(COOH)	0.592
Antibody#509	2004-07-14	2B0664	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.183
Antibody#508	2004-07-14	2B0664	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.083
*Antibody#507	2004-07-14	2B0664	MxCT(492-502)	LEVVPEDSKEL-(COOH)	0.083
Antibody#453	2003-07-22	2B1085	MxCT(226-232)	SGRDTSL-(amide)	0.400
Antibody#456	2003-07-22	2B1085	MxCT(226-232)	SGRDTSL-(amide)	0.060
Antibody#465	2003-07-22	2B1085	MxCT(256-269)	TEEVDNPEKTIPLA-(amide)	0.033
*Antibody#470	2003-07-22	2B1085	MxCT(345-356)	ASREGHLPEILS-(amide)	0.140
Antibody#471	2003-07-22	2B1085	MxCT(345-356)	ASREGHLPEILS-(amide)	0.440
*Antibody#472	2003-07-22	2B1085	MxCT(345-356)	ASREGHLPEILS-(amide)	0.370
*Antibody#473	2003-07-22	2B1085	MxCT(345-356)	ASREGHLPEILS-(amide)	0.357
Antibody#466	2003-07-22	2B1086	MxCT(226-232)	SGRDTSL-(amide)	0.307
Antibody#467	2003-07-22	2B1086	MxCT(226-232)	SGRDTSL-(amide)	0.460
Antibody#469	2003-07-22	2B1086	MxCT(226-232)	SGRDTSL-(amide)	0.470
Antibody#461	2003-07-22	2B1086	MxCT(256-269)	TEEVDNPEKTIPLA-(amide)	0.300
*Antibody#474	2003-07-22	2B1086	MxCT(345-356)	ASREGHLPEILS-(amide)	0.240
Antibody#475	2003-07-22	2B1086	MxCT(345-356)	ASREGHLPEILS-(amide)	0.467
*Antibody#476	2003-07-22	2B1086	MxCT(345-356)	ASREGHLPEILS-(amide)	0.345
*Antibody#477	2003-07-22	2B1086	MxCT(345-356)	ASREGHLPEILS-(amide)	0.420
Antibody#517	2004-07-20	3B0042	MxCT(345-356)	ASREGHLPEILS-(amide)	0.087
Antibody#514	2004-07-20	3B0045	MxCT(226-232)	SGRDTSL-(amide)	0.062
Antibody#516	2004-07-20	3B0045	MxCT(256-269)	TEEVDNPEKTIPLA-(amide)	0.032
Antibody#518	2004-07-20	3B0045	MxCT(345-356)	ASREGHLPEILS-(amide)	0.050
*Antibody#622	2006-01-27	4B0425	RxCT (1-37)	MVRKPVVATISKGGYLQGNVSGRLP SVGDQEPPGHEK-(amide)	0.092
*Antibody#618	2006-01-27	4B0447	RxCT (1-37)	MVRKPVVATISKGGYLQGNVSGRLP SVGDQEPPGHEK-(amide)	0.045
*Antibody#619	2006-01-27	4B0447	RxCT (1-37)	MVRKPVVATISKGGYLQGNVSGRLP SVGDQEPPGHEK-(amide)	0.035
Antibody#614	2006-01-27	4B0454	RxCT (1-37)	MVRKPVVATISKGGYLQGNVSGRLP SVGDQEPPGHEK-(amide)	0.420
*Antibody#616	2006-01-27	4B0454	RxCT(471-502)	DKKPKWFRRLSDRITRTLQIILEVVP EDSKEL-(COOH)	0.337
Antibody#610	2006-01-27	4B0462	RxCT (1-37)	MVRKPVVATISKGGYLQGNVSGRLP SVGDQEPPGHEK-(amide)	0.027
Antibody#612	2006-01-27	4B0462	RxCT(471-502)	DKKPKWFRRLSDRITRTLQIILEVVP EDSKEL-(COOH)	0.307
*Massie et al., 2008	2006-02-23	SB1315	MxCT(216–229)	GQTHHFKDAFSGRD-(amide)	0.206

Table 2: Commercially available antibodies that were tested in this study (* specific protein band detected).

Peptide	Manufacturer	Catalog number	Lot number	RRID	concentration (µg/ml)
HxCT between (1-50)	*Abcam plc.	ab37185	GR115623	AB_778944	2.000
HxCT between (1-50)	*Abcam plc.	ab37185	GR120606	AB_778944	0.250
HxCT between (1-50)	Abcam plc.	ab37185	GR180673	AB_778944	2.000
HxCT between (1-50)	*Abcam plc.	ab93030	24670	AB_10563683	0.250
HxCT between (1-50)	*Thermo Fisher Scientific Inc.	PA1-46264	MD1415805	AB_2190850	0.500
HxCT between (1-50)	*Thermo Fisher Scientific Inc.	PA1-16893	OB1663384	AB_2286208	2.000
HxCT between (1-50)	Thermo Fisher Scientific Inc.	PA1-16893	PH1894974	AB_2286208	0.333
HxCT between (1-50)	*Novus Biologicals	NB 300-318	G3	AB_527560	0.200
HxCT between (1-50)	Novus Biologicals	NB 300-318	H1	AB_527560	0.500
MxCT N-terminal region	Transgenic Inc.	KE021	TG210612	AB_1627148	1.000

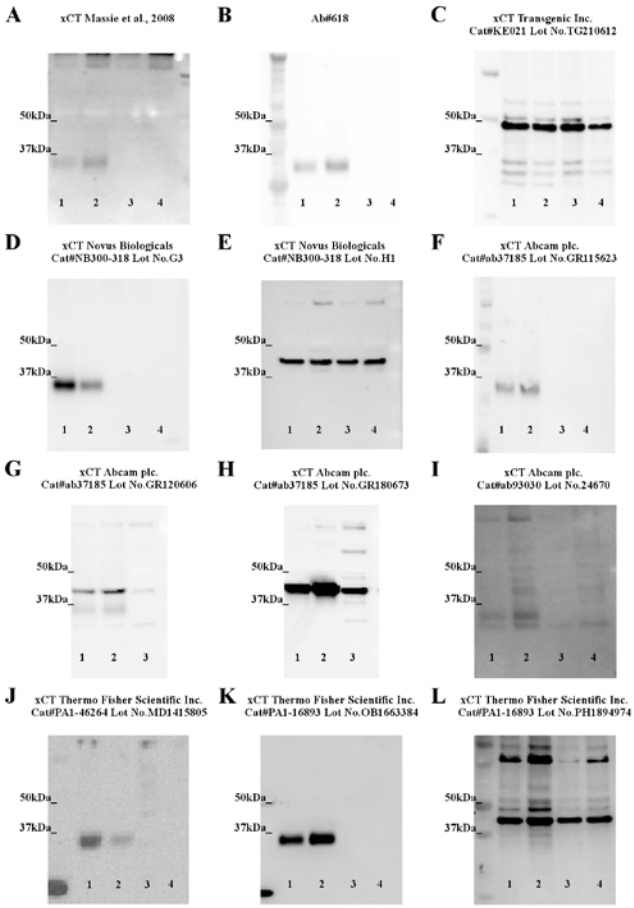

Figure 3: Specificity of anti-xCT antibodies: representative examples of the different Western blot experiments (2 concentrations of each sample were loaded; lanes 1-2: $xCT^{+/+}$ mouse brain tissue samples; lanes 3-4: $xCT^{-/-}$ mouse brain tissue samples).

After incubation with anti-xCT antibodies: Massie et al., 2008 **(A)** and Ab#618 **(B)** and commercial antibodies: Novus Biologicals Cat# NB 300-318 Lot No.G3 **(D)**, Abcam plc. Cat#ab37185 Lot No.GR115623 **(F)**, Abcam plc. Cat#ab37185 Lot No.GR120606 **(G)**, Abcam plc. Cat#ab93030 Lot No.24670 **(I)**, Thermo Fischer Scientific Inc. Cat#PA1-46264 Lot No.MD1415805 **(J)** and Thermo Fisher Scientific Inc. Cat#PA1-16893 Lot No.OB1663384 **(K)** specific xCT bands are observed of about 35 kDa in the $xCT^{+/+}$ samples which are absent in the $xCT^{-/-}$ samples.

No specific bands are discernable after incubation with the commercial anti-xCT antibodies: Transgenic Inc. Cat#KE021 Lot No.TG210612 **(C)**, Novus Biologicals Cat# NB 300-318 Lot No.H1 **(E)**, Abcam plc. Cat#ab37185 Lot No.GR180673 **(H)** and Thermo Fisher Scientific Inc. Cat#PA1-16893 Lot No.PH1894974 **(L)**. These antibodies give rise to a nonspecific protein band of about 40-50 kDa present in both $xCT^{+/+}$ and $xCT^{-/-}$ brain homogenates.

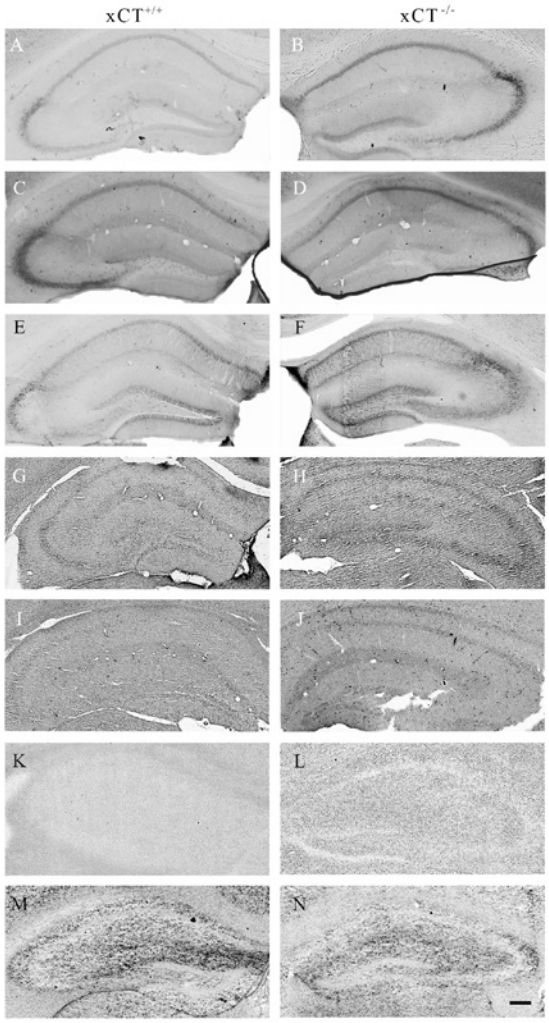

Figure 4: Specificity of anti-xCT antibodies: representative examples of distinct immunohistochemical experiments (left panels: xCT+/+ mouse brain samples; right panels: xCT-/- mouse brain samples).
(**A-B**) Cardiac perfusion with 4% formaldehyde (F) and incubation with our own xCT antibody Massie et al. 2008, trypsine digestion and Tris-buffered saline (TBS, 0,01M, 0,1% TritonX-100, pH 7,4) rinsing steps.
(**C-D**) Cardiac perfusion with 4% F and incubation with Thermo Fisher Scientific Inc. Cat#PA1-16893 Lot No.OB1663384, TBS (0,03% TritonX-100) rinsing steps.
(**E-F**) Cardiac perfusion with 4% F and incubation with Abcam plc. Cat#ab37185 Lot No.GR115623, phosphate-buffered saline (PBS) rinsing steps.
(**G-H**) Cardiac perfusion with 4% F, serial sucrose incubation (15-20-30%), snap-freezing and incubation with Abcam plc. Cat#ab37185 Lot No.GR115623.
(**I-J**) Cardiac perfusion with a mixture of 4% F and 0.35% glutaraldehyde, 48h incubation in 15% sucrose, snap freezing and incubation with Transgenic Inc. Cat#KE021 Lot No.TG210612.
(**K-L**) Snap-freezing, 10 min post-fixation in 4% F and incubation with Ab#618.
(**M-N**) Snap-freezing, 10 min post-fixation in ice-cold acetone and incubation with Novus Biologicals Cat# NB 300-318 Lot No.G3.
Scale bar in N = 250µm (applies to all panels)

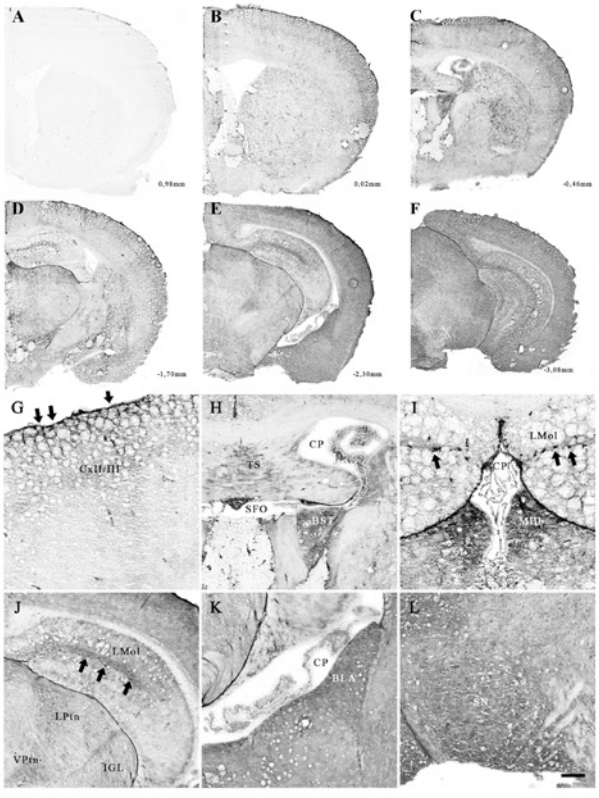

Figure 5: Regional distribution of xCT throughout the mouse forebrain, as determined using the Ab#618 antibodies to xCT. (**A-F**) Immunohistochemical labeling at distinct levels in the $xCT^{+/+}$ mouse brain (**B-F**) is absent in the $xCT^{-/-}$ mouse brain (**A**). Coordinates relative to bregma are depicted at right bottom. (**G-L**) Structures in $xCT^{+/+}$ mouse brain showing dense xCT immunoreactivity. **G**: leptomeninges (arrows), cortical layers II-III (CxII/III), **H**: subfornical organ (SFO), bed nucleus of the stria terminalis (BST), triangular septal nucleus (TS), choroid plexus (CP), **I**: choroid plexus (CP), medial habenular nucleus (MHb), lacunosum moleculare layer of the hippocampus (LMol), hippocampal vasculature (black arrows), **J**: lacunosum moleculare layer of the hippocampus (LMol), lateral posterior thalamic nucleus (LPtn), ventral posteromedial thalamic nucleus (VPtn), intergeniculate leaf (IGL), hippocampal vasculature (black arrows), **K**: choroid plexus (CP), basolateral amygdala (BLA), L: substantia nigra (SN). Scale bar in L = 175µm applies to G, I, L; 750µm for A-F; 375µm for H, J; 250µm for K.

6. Neuroprotective effects of zonisamide against lactacystin-induced neurodegeneration are independent of system x_c^-.

Zonisamide (ZNS), used in symptomatic treatment of PD[36-38], was recently reported to exert neuroprotection in rodent models[39-42]. One of the proposed neuroprotective mechanisms involves elevated xCT protein expression, inducing GSH synthesis[40]. This is however in contrast with our hypothesis that inhibition of system x_c^- might be a neuroprotective strategy for the treatment of PD[19]. Therefore, we investigated the outcome of ZNS treatment in a mouse model of PD based on intranigral proteasome inhibition, and whether the observed effects would be mediated by system x_c^-. The proteasome inhibitor LAC (or vehicle, Sham) was administered intranigrally to mice receiving repeated i.p. injections of either ZNS 30mg/kg or vehicle (placebo). Drug administration was initiated three days prior to stereotaxic LAC injection and was maintained until six days post surgery. One week after lesion, mice were behaviorally assessed and investigated in terms of nigrostriatal neurodegeneration and molecular changes at the level of the basal ganglia, including expression levels of xCT. In a separate set of experiments, the impact of ZNS treatment on system x_c^- was investigated in naive, control conditions *in vivo* as well as *in vitro*. ZNS reduced the loss of nigral dopaminergic neurons following LAC infusion (fig. 6) and the degree of sensorimotor impairment (fig. 7). ZNS failed, however, to modulate xCT expression in basal ganglia of lesioned mice (fig. 8). Similarly, ZNS did not influence xCT or GSH levels in naive mice (fig. 9), nor did it alter system x_c^- activity or GSH content *in vitro* (fig. 10). Taken together, these results demonstrate that ZNS treatment provides neuroprotection and behavioral improvement in a PD mouse model based on proteasome inhibition, via system x_c^- independent mechanisms. These results have been **submitted to Neuropharmacology** (Bentea *et al.*, under review).

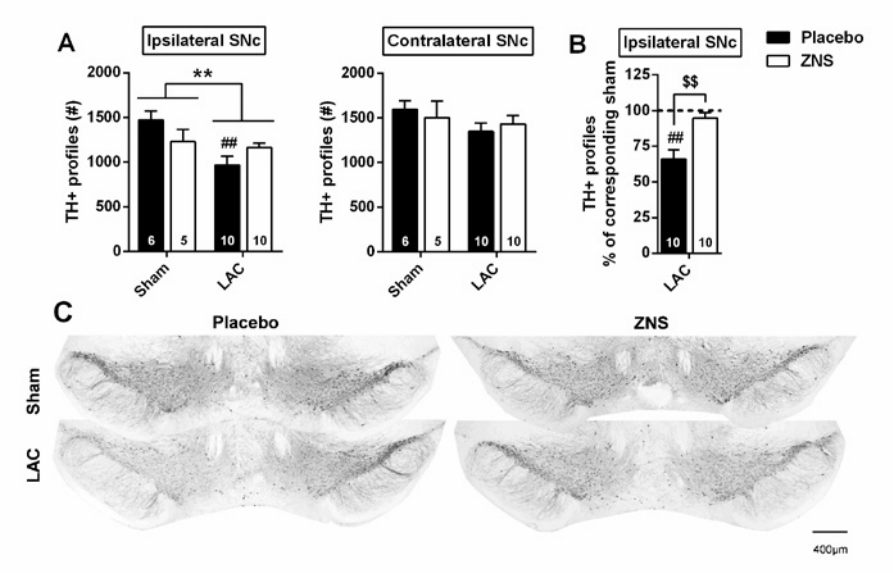

Figure 6: Repeated administration of zonisamide (ZNS) protects against lactacystin (LAC)-induced nigral neurodegeneration. (**A**) Tyrosine hydroxylase (TH) immunohistochemistry revealed a global loss of TH+ neurons after LAC lesion in the ipsilateral substantia nigra pars compacta (SNc). Significant loss of TH+ profiles was observed in the placebo-treated group, compared to their corresponding sham group. (**B**) ZNS treatment reduced the LAC-induced loss of SNc TH+ profiles. Bars represent mean ± SEM, **p<0.01 (two-way ANOVA), ##p<0.01 (Tukey's post-hoc comparison to corresponding sham), $$p<0.01 (Tukey's post-hoc comparison to placebo-treated). Sample sizes are mentioned in the bars. (**C**) Representative TH photomicrographs of the SNc of the four groups of mice. Scale bar 400 µm.

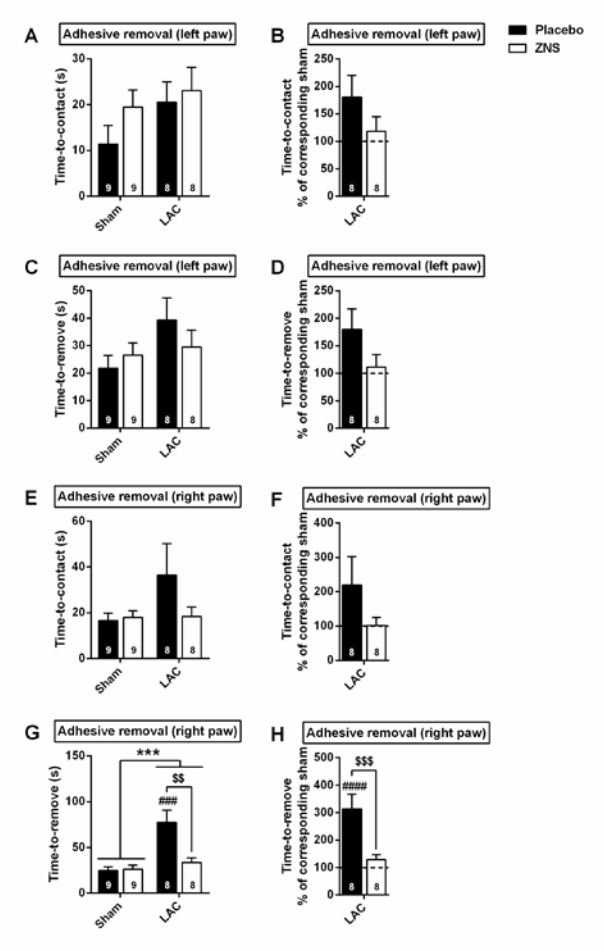

Figure 7: Repeated zonisamide (ZNS) treatment improves sensorimotor performance of lactacystin (LAC)-lesioned mice. (**A**-**D**) In the adhesive removal test, time-to-contact and time-to-remove the adhesive strips remained unaltered on the ipsilateral side. (**E**-**H**) On the contralateral side, LAC lesion induced a significant delay in the time-to-remove that was effectively reduced by ZNS treatment. Bars represent mean ± SEM, ***p<0.001 (two-way ANOVA), ###p<0.001, ####p<0.0001 (Tukey's post-hoc comparison to corresponding sham), $$p<0.01, $$$p<0.001 (Tukey's post-hoc comparison to placebo-treated). Sample sizes are mentioned in the bars.

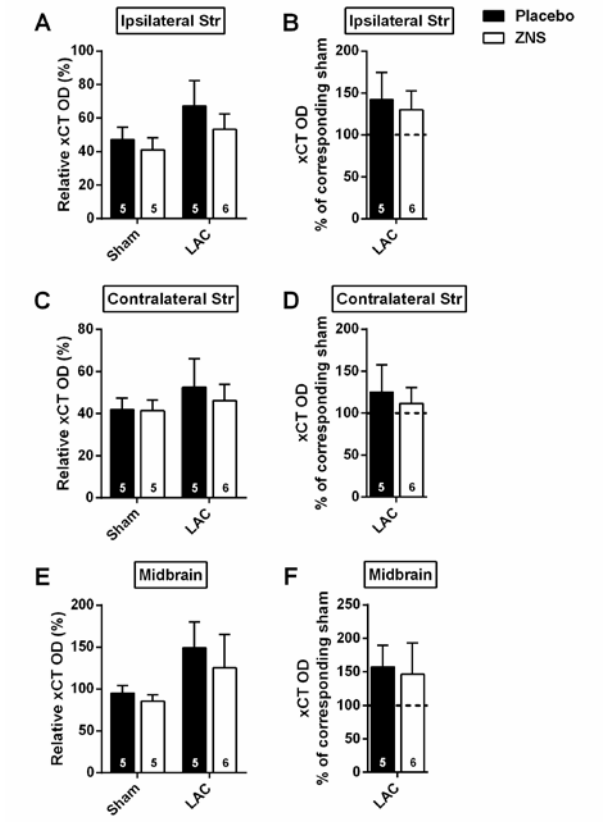

Figure 8: Repeated administration of zonisamide (ZNS) does not influence xCT protein expression one week after lactacystin (LAC) administration. xCT protein expression was not affected by LAC-lesion nor ZNS treatment, both in striatum (**A**-**D**) and midbrain (**E**, **F**). Bars represent mean ± SEM. Sample sizes are mentioned in the bars.

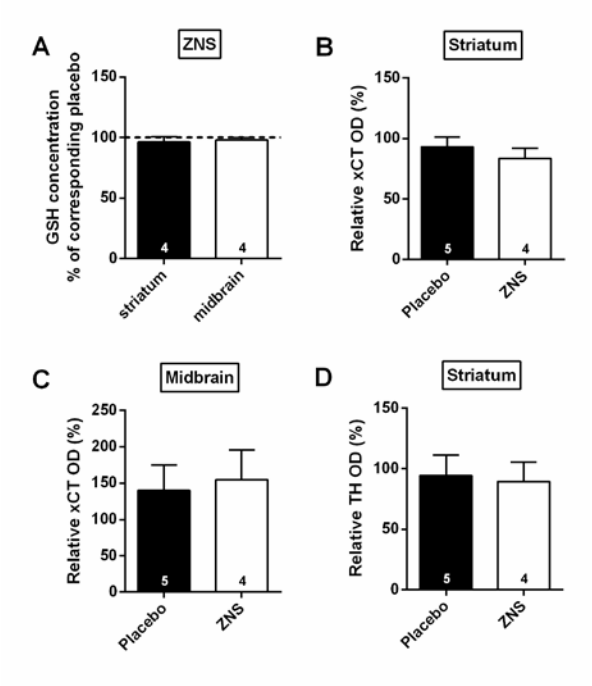

Figure 9: Repeated administration of zonisamide (ZNS) to naïve mice does not influence glutathione (GSH) or xCT expression levels in the basal ganglia. (**A**) No effect of a 14 day ZNS treatment on midbrain and striatal GSH levels in $xCT^{-/-}$ mice and their $xCT^{+/+}$ littermates. (**B**, **C**) Quantification of striatal (**B**) and midbrain (**C**) xCT expression levels by Western blotting in $xCT^{+/+}$ mice after ZNS treatment failed to reveal any significant difference compared to placebo controls. (**D**) Repeated administration of ZNS did not influence striatal protein expression of TH. Bars represent mean ± SEM. Sample sizes are mentioned in the bars.

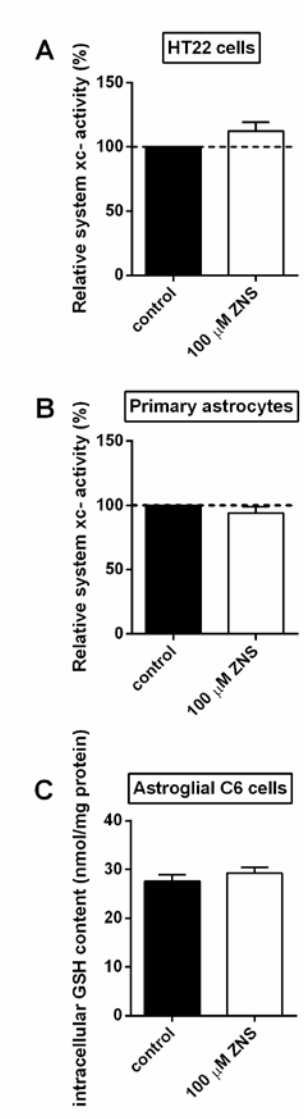

Figure 10: Zonisamide (ZNS) does not influence the activity of system x_c^- or intracellular glutathione (GSH) levels *in vitro*. (**A**-**C**) Lack of effect of 100 µM ZNS administration on relative system x_c^- activity in HT22 cells upon 24 h of ZNS (**A**) and microglia-free murine primary astrocytes upon 48 h of ZNS (**B**), and on intracellular GSH content in astroglial C6 cells upon 24 h of ZNS (**C**). Bars represent mean ± SEM of six (**A**) and five (**B**, **C**) independent experiments.

7. Summary of the main achievements/findings in the first two years (2014-2015) of this project:

1. In-depth analysis of the specificity problem of anti-xCT antibodies
2. Development and characterization of a new intranigral LAC mouse model for PD, showing α-synuclein accumulation as well as motor and non-motor impairment
3. Behavioral phenotyping of the xCT$^{-/-}$ mice, with clear anxiolytic and anti-depressive like effects
4. Further evidence for inhibition of system x_c^- as potential neuroprotective strategy for the treament of PD, without negative effects in the elder and possibly beneficial for neuro-psychiatric co-morbidities
5. Confirmation of system x_c^- - independent, neuroprotective effects of ZNS in the LAC model

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

- Bentea E, Van Liefferinge J, Martens K, Kobayashi S, Deneyer L, Verbruggen L, Demuyser T, Albertini G, Maes K, Sato H, Smolders I, Lewerenz J, Massie A. Zonisamide reduces lacatcystin-induced nigral dopaminergic neurodegeneration and sensorimotor impairment in mice via system x_c^- independent mechanisms. Neuropharmacology, submitted
- Demuyser T, Deneyer L, Bentea E, Albertini G, Van Liefferinge J, Merckx E, De Prins A, De Bundel D, Massie A, Smolders I (2016) In-depth behavioral characterization of the corticosterone mouse model and the critical involvement of housing conditions. Physiology & Behavior, in press.
- Massie A, Boillée S, Hewett S, Knackstedt L, Lewerenz J (2015) Main path and byways : non-vesicular glutamate release via system x_c^- as an important modifier of glutamatergic neurotransmission. Journal of Neurochemistry, in press.
- Van Liefferinge J, Bentea E, Demuyser T, Albertini G, Follin-Arbelet V, Holmseth S, Merckx E, Sato H, Aerts J, Smolders I, Arckens L, Danbolt NC, Massie A (2015) Comparative analysis of antibodies to xCT (Slc7a11) : forewarned is forearmed. Journal of Comparative Neurology, in press.
- El Arfani A, Albertini G, Bentea E, Demuyser T, Van Eeckhaut A*, Smolders I*, Massie A* (2015) Alterations in the motor cortical and striatal glutamatergic and D-serinergic system in the bilateral 6-hydroxydopamine rat model for Parkinson's disease. Neurochemistry International 88: 88-96. (*equally contributing authors)
- 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* (2015) 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 59: 49-58. (*equally contributing authors)
- 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 (2015) Nigral proteasome inhibition in mice leads to parkinsonism including non-motor impairment and alpha-synuclein phosphorylation at Ser129. Frontiers in Behavioral Neuroscience 9: 68.
- Bentea E*, Sconce MD*, Churchill MJ*, Van Liefferinge J, Sato H, Meshul CK, Massie A (2015) MPTP-induced parkinsonism in mice alters striatal and nigral xCT expression but is unaffected by the genetic loss of xCT. Neuroscience Letters, Neuroscience Letters 593: 1-6. (*equally contributing authors)
- 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

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

- De Bundel D*, Fafouri A*, Csaba Z*, Loyens E, Lebon S, El Ghouzzi, V, Peineau S, Vodjdani G, Kiagiadaki F, Aourz N, Coppens J, Walrave L, Portelli J, Vanderheyden P, Chai SY, Thermos K, Bernard V, Collingridge G, Auvin S, Gressens P, Smolders I*, Dournaud P* (2015) Trans-modulation of the somatostatine type 2 receptor trafficking by insulin-regulated aminopeptidase decreases limbic seizures. Journal of Neuroscience 35: 11960-75.
- Van Liefferinge J, Jensen C, Albertini G, Bentea E, Demuyser T, Merckx E, Aronica E, Smolders I, Massie A (2015) Altered vesicular glutamate transporter expression in human temporal lobe epilepsy with hippocampal sclerosis. Neuroscience Letters 590: 184-8.

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Deciphering the role of protein acetylation in primary ciliogenesis

1. Background and significance

Cilia are subdivided into two categories : motile cilia (secondary cilia) that enable fluid movement over epithelial surfaces and primary cilia that are localized at the surface of most cell types during G0 and act as a "sensor" to transduce extracellular signaling, thus contributing to a broad array of homeostatic mechanisms (Corbit et al., 2005; Singla and Reiter, 2006; Rohatgi et al., 2007; Berbari et al., 2009). The primary cilium is nucleated from and anchored to a BB, which is composed of an axoneme enriched in microtubules (MT) surrounded by a ciliary membrane connected to the plasma membrane. A transition zone between the BB and the axoneme acts as molecular barrier and docking station for intraflagellar transport (IFT) and motor proteins (Pedersen and Rosenbaum, 2008). Despite our growing understanding of its biological role, we do not know much about the factors that contribute to its assembly and positioning at the cell membrane during brain development. Accumulating evidence shows that mutations in cilia genes can lead to malformations underlying neurological or psychiatric disorders, as well as deafness and balance disorders (Norris and Grimes, 2012).

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 (Choudhary et al., 2009). Although it was until recently exclusively associated with transcriptional activation (through neutralization of positive charges of core histone tails lysines(Ren and Gorovsky, 2001)), there is now growing evidences to support lysine acetylation of a broad range of non-histone proteins(Choudhary and Grant, 2004; Kim et al., 2006; Close et al., 2010). 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(Spange et al., 2009). Recent studies suggest that acetylation of cytoplasmic substrates contributes to brain development(Reed et al., 2006; Creppe et al., 2009) and, that disruption of this process is associated with various progressive neurological disorders(Hempen and Brion, 1996; Dompierre et al., 2007). 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(Slaugenhaupt et al., 2001; Slaugenhaupt and Gusella, 2002). Elongator comprises six subunits (Elp1-6). Elp1 is a scaffold subunit required for functional assembly of Elongator and Elp3 acts as DNA demethylase(Okada et al., 2010) and a KAT. This multiprotein complex promotes Nε-acetylation of histone H3 lysines(Winkler et al., 2002), 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(Creppe et al., 2009; Solinger et al., 2010) and Bruchpilot (Miskiewicz et al., 2011). In addition, Elongator activity in animals 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 (Creppe et al., 2009; Laguesse et al., 2015). Our preliminary data support a novel function of Elongator in primary ciliogenesis in the perinatal subventricular zone and at the kinocilium (derived form primary cilium) in hair cell progenitors. Our aim is to uncover the role of acetylation in primary (kino)-ciliogenesis by addressing the role of Elongator as an entry point.

2. Elongator controls primary ciliogenesis in the developing brain

The coordinated directional beating of multi-ciliated ependymal cells that board the surface of the lateral ventricle (LV), creates a unidirectional CSF flow named ependymal flow (Miyan et al., 2003), which is essential for brain homeostasis and olfactory bulb neurogenesis (Sawamoto et al., 2006). Disruption of ciliogenesis (e.g. primary ciliopathies such as Joubert's syndrome and Meckel-Gruber syndrome) or ependymal cell polarization often result in hydrocephalus and brain malformations(Badano et al., 2006; Mirzadeh et al., 2010; Vogel et al., 2012; Lee, 2013; Boutin et al., 2014; Ohata et al., 2014). Ependymal cells are polarized along the apico-basal axis as well as also along the epithelial plan through a process called planar cell polarity (PCP). Establishment of PCP during ependymal cell maturation is an essential prerequisite for the coordinated ciliary beating across the lateral wall after birth (Guirao et al., 2010; Mirzadeh et al., 2010; Boutin et al., 2014; Ohata et al., 2014). PCP was originally identified in *Drosophila* and is characterized by the planar-polarized accumulation of PCP core components (in mammals: atypical seven-pass transmembrane cadherin; Celsr (Celsr1-3), Frizzled3 (Fzd3), Frizzeled6 (Fzd6), Van Gogh like1-2 (Vangl1-2), Disheveled1-3 (Dvl1-3), and Prickle like 1–4 (PK1-4)) to distinct regions of the cell. Their mutations impair primary cilium development and function, linking ciliogenesis with planar polarity and hydrocephalus (Tissir et al., 2010; Boutin et al., 2014; Ohata et al., 2014; Sowers et al., 2014; Ohata et al., 2015). In ependymal cells, motile cilia nucleate from microtubule-based structures/platform called basal bodies (BBs), which are the first determinants of ependymal cell polarity. Planar cell polarity underlies different biological events (Mirzadeh et al., 2010) : (1) rotational polarity, which corresponds to the unidirectional alignment of motile cilia in the cell and the coordination of this process at the tissue level (Boutin et al., 2014; Ohata et al., 2014); and (2) translational polarity, which refers to the migration and the positioning of the cluster of BBs (and their corresponding cilia) in ependymal cells (Guirao et al., 2010). Moreover, radial glial cells (RGCs), which are the ependymal cell progenitors (Spassky et al., 2005), already show horizontal migration of their primary cilia, suggesting that ependymal cell polarity establishment is a gradual process starting in their progenitors where the primary cilium plays a central role for establishing intrinsic as well as tissular cell polarity (Ross et al., 2005; Park et al., 2006; Wallingford, 2006; Jones et al., 2008; Mirzadeh et al., 2010).

By coupling genetic, histological and biochemical approaches, we identified Elp3, the catalytic subunit of the Elongator complex, as a regulator of primary ciliogenesis and PCP establishment in the lateral wall of the postnatal mouse brain. Immunolabelings uncovered Elp1 and Elp3 expression at peri-centriolar material (PCM)/basal body and/or transition zone. We crossed FoxG1:Cre mice (Hebert and McConnell, 2000) with Elp3 lox/lox mice to conditionally delete *Elp3* from telencephalon progenitors (Elp3 cKO mouse), including radial glia cells. Our preliminary results showed that the conditional loss of Elp3 in forebrain progenitors leads to postnatal hydrocephalus in mice. 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 performed 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.

Functional analyses demonstrated that Elongator is required for proper growth and positioning of the primary cilium of RGCs as well as the establishment of the PCP in maturating ependymal cells of the lateral wall in mouse. This phenotype results from the activation the PERK/Atf4 pathway, which may further disturbs Notch signaling via blockade of its intracellular furin-dependant clivage and thus membrane maturation. These results provide a putative link between Elp3 and Notch signaling during ciliogenesis and polarity establishment in the lateral wall of the postnatal brain. We are currently validating this hypothesis and a manuscript will be soon submitted.

3. 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 (Jones et al., 2008; Sipe and Lu, 2011).Our preliminary results suggest that in the absence of Elp3 the kinocilium is shorter and PCP defects are present in the inner ear. 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.

3.1. 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.
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 (Hasson et al., 1997; Wolfrum et al., 1998), 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 VIIA or Ic sequence and if such domains are present, we will perform microtubule-binding assays as previously described(Koonce and McIntosh, 1990) 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.

3.2. 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 confirm PCP defects, membrane recruitment and asymmetric localization of core PCP proteins have been studied. We found that Elp3 is necessary for membrane recruitment and asymetric position of dishevelled 2 (Dvl2), Vangl1 and vangl2, three core PCP proteins previously identified in the developing inner ear (Ezan and Montcouquiol, 2013). These results confirm that Elp3 is necessary for a correct development of the cochlea. We are now studying if those proteins are direct targets of Elp3 by studying if these proteins are acetylated.

We also found that Elp3 has a very important role in intrinsic polarity of hair cells. We found that aPKC is normally laterally positioned is diffusely present in the cytoplasm. This has been recently confirmed for another protein, cytoplasmic dynein. In parallel, we observed that the kinocilium is significantly mispositioned in cochlear hair cells from Elp3 deficient mice. Taken together, these findings suggest that Elp3 cooperates with dynein and aPKC to regulate kinocilium positioning in hair cells. We will study if Elp3 interacts with aPKC and/or dynein by inducing their acetylation that is necessary for stabilizing these proteins.

3.3. 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(Baltus et al., 2009). 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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- Laguesse, S., Creppe, C., Nedialkova, D., Prevot, P.-P., Borgs, L., Huysseune, S., Franco, B., Duysens, G., Krusy, N., Lee, G., Thelen, N., Thiry, M., Close, P., Chariot, A., Malgrange, B., Leidel, S., Godin, J., and, **Nguyen, L**. : A dynamic unfolded protein response contributes to the control of cortical neurogenesis.
  Dev Cell (2015), 35(5): 553-567 (I.F. 2014= 9.708)
- Peyre, E., Silva, C., and **Nguyen, L**.: Crosstalk between intracellular and extracellular signals regulating interneuron production, migration and integration into the cortex
  Front Cell Neurosci (2015), (9, #129): 1-18 (I.F. 2013= 4.175)
- Huyghe, A., Van den Ackerveken, P., Sacheli, R., Prevot, P.-P., Thelen, N., Renault, J., Thiry, M., Delacroix, L., **Nguyen, L**., and Malgrange, B. : MicroRNA-124 dependent modulation of Wnt signaling pathway regulates cell fate specification in the cochlea.
  Cell Rep (2015), 13(1): 31-42 (I.F. 2014=8.358)

6. Other publications of the laboratory in 2015

- Ladang, A., Rapino, F., Heukamp, L., Tharun, L., Shostak, K., Hermand, D., Delaunay, S., Klevenic, I., Jiang, Z., Jacques, N., Jamart, D., Migeot, V., Florin, A., Göktuna, S., Malgrange, B., Sansom, O., **Nguyen, L**., Büttner, R., Close, P., and Chariot, A. : Elp3 drives Wnt-dependent tumor initiationand regenerationin the intestine.
  J Exp Med (2015), 212(12): 2057-75 (I.F. 2014=12.515)

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

Alzheimer's disease (AD) is a biologically complex dementia characterized by a slow and progressive loss of neurons in the brain. A clear correlation exists between clinical signs of dementia and the presence in the brain of abnormal protein deposits, which are made of intraneuronal hyperphosphorylated protein tau accumulating in neurofibrillary tangles. In AD, extracellular deposits are also found in senile plaques, which contain aggregated amyloid peptide Aβ, which is produced from the amyloid precursor protein APP. With the discovery that APP mutations can act as fully penetrant AD genes in rare inherited AD cases, the amyloid cascade hypothesis was proposed. In this hypothesis, an imbalance in Aβ production and clearance leads to gradual accumulation and aggregation of the peptide in the brain, initiating a neurodegenerative cascade that involves amyloid deposition, inflammation, oxidative stress and neuronal loss. Oligomeric Aβ causes long term potentiation (LTP) impairment and synaptic dysfunction and accelerates the formation of neurofibrillary tangles that cause synaptic failure and neuronal death. In contrast to the rarity of inherited cases, the sporadic form of AD is quite prevalent and there are growing amounts of data, including a number of failed clinical trials, suggesting that the amyloid cascade hypothesis is insufficient. A quarter to a third of all older individuals with normal cognitive function have a detectable amyloid burden by PET scanning, indicating that it is possible to have amyloid deposits without dementia. On the other hand, individuals who entered anti-amyloid antibody clinical trials were diagnosed with AD, and have still AD although their plaque burden has been dramatically reduced. The implication is that just as there can be plaques without AD, there can also be AD without plaques.

Considering that the amyloid cascade hypothesis cannot explain the complexity of the dementing illness, an alternative approach that we developed several years ago was to focus on the function of APP in order to investigate whether APP dysfunction could contribute to AD pathology.

Polymorphisms in genes encoding proteins involved in cholesterol homeostasis are associated with AD, and abnormal accumulation of cholesterol has been demonstrated in post mortem AD brain, arguing that perturbation of cholesterol homeostasis favors progression of AD. Neuronal cholesterol homeostasis is known to be essential for basic synaptic function, plasticity and behavior and we recently demonstrated that APP controls cholesterol turnover needed for neuronal activity.

The beneficial effects of Retinoic X Receptor (RXR) agonists reported on synaptic and cognitive functions are based on their ability to increase cholesterol efflux, further emphasizing the stimulation of neuronal cholesterol turnover as a possible target for the treatment of dementia. We studied the effects of Targretin® (bexarotene) on cognition and biomarkers in a patient with mild AD. Targretin® is a RXR agonist shown to improve synaptic and cognitive functions in animal models of AD by increasing neuronal cholesterol efflux. After 6 months of treatment with Targretin® 300 mg/day, memory improved by about 40% and the tau protein in the cerebrospinal fluid decreased by about 20%. No significant side effects were noticed. This observation in a single patient indicates that Targretin® may improve memory performance and biological markers at an early stage of AD. These results are in press in the "Journal of Alzheimer's Disease".

Besides its crucial role in the pathogenesis of AD, the knowledge of APP physiological functions remains surprisingly scarce. We observed that APP regulates the transcription of the Glial cell line-Derived Neurotrophic Factor (GDNF). APP-dependent regulation of GDNF expression affects muscles strength, muscular trophy and both neuronal and muscular differentiation fundamental for neuromuscular junctions (NMJs) maturation in vivo. In a nerve-muscle co-culture model set up to modelize NMJs formation *in*

vitro, silencing of muscular APP induces a 30% decrease in secreted GDNF levels and a 40% decrease in the total number of NMJs together with a significant reduction in the density of acetylcholine vesicles at the presynaptic site and in neuronal maturation. These defects are rescued by GDNF expression in muscle cells in the conditions where muscular APP has been previously silenced. Expression of GDNF in muscles of APP null mice (APP-/-) corrected the aberrant synaptic morphology of NMJs. Our findings highlight for the first time that APP-dependent GDNF expression drives the process of NMJs formation, providing new insights into the link between APP gene regulatory network and physiological functions. These results are in press in the "FASEB Journal"

The proinflammatory cytokine interleukin-1β (IL-1β) is overexpressed in AD as a key regulator of neuroinflammation. Aβ peptide triggers activation of inflammasomes, protein complexes responsible for IL-1β maturation in microglial cells. Downregulation of NALP3 inflammasome has been shown to decrease amyloid load and rescue cognitive deficits in a mouse model of AD. Whereas activation of inflammasome in microglial cells has been described in AD, no data are currently available concerning activation of inflammasome in astrocytes, although they are involved in inflammatory response and phagocytosis. By targeting the inflammasome adaptor protein ASC, we investigated the influence of activation of the inflammasome on the phagocytic activity of astrocytes. We used an ASC knockout mouse model, as ASC is a central protein in the inflammasome, acting as an adaptor and stabilizer of the complex and thus critical for its activation. Lipopolysaccharide-primed primary cultures of astrocytes from newborn mice were utilized to evaluate Aβ-induced inflammasome activation by measuring IL-1β release by ECLIA (Electro-chemiluminescence immunoassay). Phagocytosis efficiency was measured by incorporation of bioparticles, and the release of the chemokine CCL3 was measured by ECLIA. ASC mice were crossbred with 5xFAD mice, a well characterized model of AD, and tested for spatial reference memory using the Morris water maze at 7-8 month of age. Amyloid load and CCL3 were quantified by thioflavine S staining and qRT-PCR, respectively. Cultured astrocytes primed with LPS and treated with Aβ showed an ASC dependent production of IL-1β resulting from inflammasome activation mediated by Aβ phagocytosis and cathepsin B enzymatic activity. ASC+/- astrocytes displayed a higher phagocytic activity as compared to ASC+/+ and ASC -/- cells, resulting from a higher release of the chemokine CCL3. A significant decrease in amyloid load was measured in brain of 7-8 month-old 5xFAD mice carrying the ASC +/- genotype, correlated with an increase in CCL3 gene expression. In addition, the ASC +/- genotype rescued spatial reference memory deficits observed in 5xFAD mice. These results demonstrate that Aβ is able to activate astrocytic inflammasome. Downregulation of inflammasome activity increases phagocytosis in astrocytes due to release of CCL3. This could explain why downregulation of inflammasome activity decreases amy-loid load and rescues memory deficits in a mouse model of AD. These results are in press in the "Journal of Neuroinflammation".

1. Results in press in 2015 thanks to the FMRE.

1. Targretin improves cognitive and biological markers in a patient with Alzheimer's disease.
   Nathalie Pierrot, Renaud Lhommel, Lisa Quenon, Bernard Hanseeuw, Laurence Dricot,
   Christian Sindic, Jean-Marie Maloteaux, Jean-Noël Octave and Adrian Ivanoiu.
   Journal of Alzheimer's Disease. I.F. 4.151
2. APP-dependent Glial cell line-Derived Neurotrophic Factor (GDNF) gene expression drives neuromuscular junctions formation.
   Serena Stanga, Nadège Zanou, Emilie Audouard, Bernadette Tasiaux, Sabrina Contino,Gaëlle Vandermeulen, Frédérique René, Jean-Philippe Loeffler, Frédéric Clotman, Philippe Gailly, Ilse Dewachter, Jean-Noël Octave and Pascal Kienlen-Campard.
   FASEB Journal. I.F. 5.043
3. Activation of phagocytic activity in astrocytes by reduced expression of the inflammasome component ASC and its implication in a mouse model of Alzheimer disease.
   Julien Couturier, Ilie-Cosmin Stancu, Olivier Shackman, Nathalie Pierrot, François Huaux, Pascal Kienlen-Campard, Ilse Dewachter, Jean-Noël Octave.
   Journal of Neuroinflammation. I.F. 5.41

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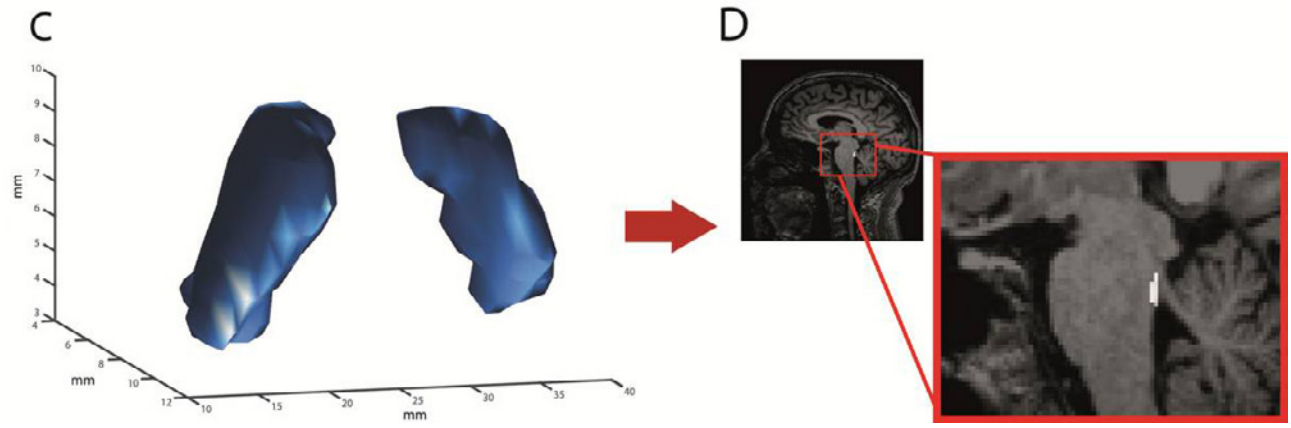
Parkinson's disease revisited. A new vision of basal ganglia functions in the context of the Parkinson's disease



1. Introduction

Parkinson's disease (PD) is the second most frequent neurodegenerative disease, caused by a neuronal loss in the pars compacta of Substantia Nigra (SNc), leading to a depletion of dopamine in the striatum. PD is characterized by well-known motor symptoms, namely bradykinesia, rigidity, resting tremor and postural instability, although non-motor symptoms, such a sleep and emotional disorders, are increasingly recognized as an integral part of the clinical picture of PD. However, despite decades of extensive investigation, the precise role of the basal ganglia (BG) to motor and non-motor functions, and their contribution to PD symptoms, are still under heated debates.

Indeed, the exact link between the dopamine depletion in the striatum and motor and non-motor symptoms of PD remains puzzling. Recently, it has been suggested that the loss of dopaminergic neurons in the SNc could affect the reward and decision-making processes and, thus, 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.

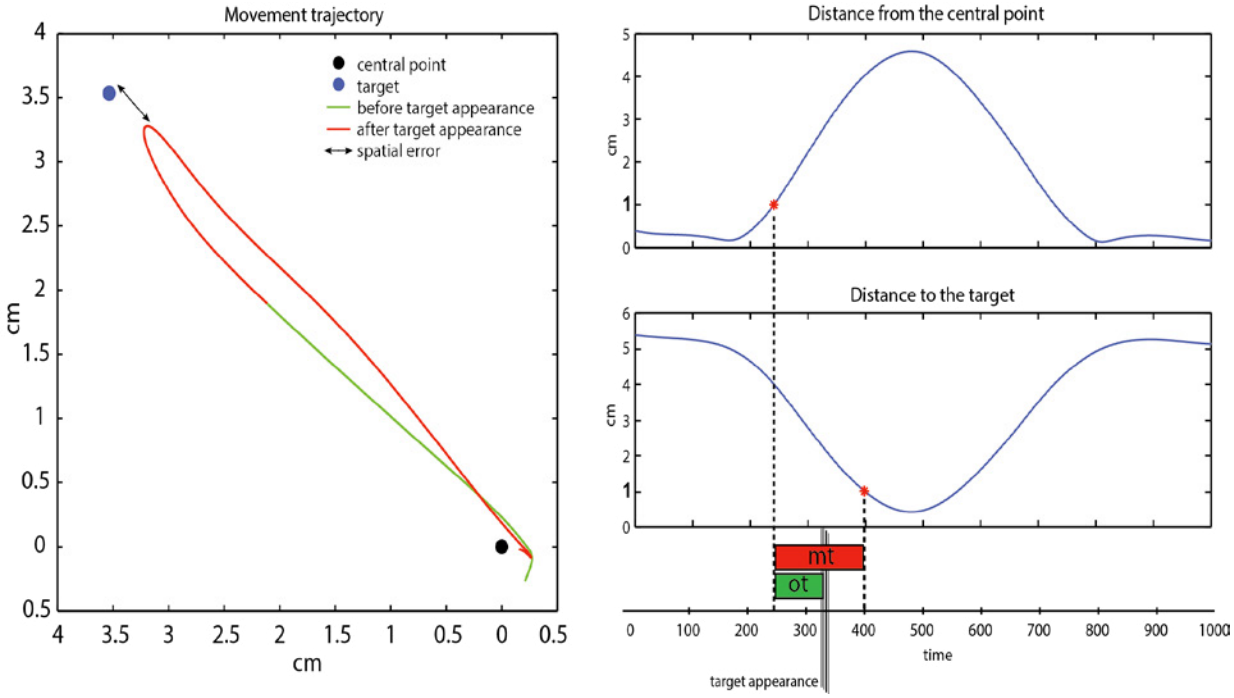
Amongst the motor functions assigned to the BG, a significant one is the storage and recall of overlearned sequential skills. Interestingly, it has been shown recently that the putamen, a part of the striatum, is recruited during chunking, a mechanism which permits to group discrete movements as a single complex action. To determine the neural correlates of chunking, and the contribution of BG to chunking in particular, is the second main question addressed in this project.

Finally, as aforementioned, non-motor symptoms of PD remain largely under-appreciated and under-investigated, although some of them have a significant negative impact on the quality of life of PD patients. Because these non-motor symptoms have a relatively poor response to dopamine replacement therapy, they may involve the serotonergic and noradrenergic systems. One of the most common non-motor symptoms associated with PD is fatigue and interestingly, it has been show that the locus coeruleus – which plays a key role in arousal and is the main source of noradrenaline in the central nervous system – is involved at an earlier (pre-motor) stage of the PD than the SNc (Braak et al., 2003). Investigating these non-motor symptoms may open a possibility to identify individuals at risk in the population.

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

2.1. Chunking as a cost saving strategy in motor skill learning

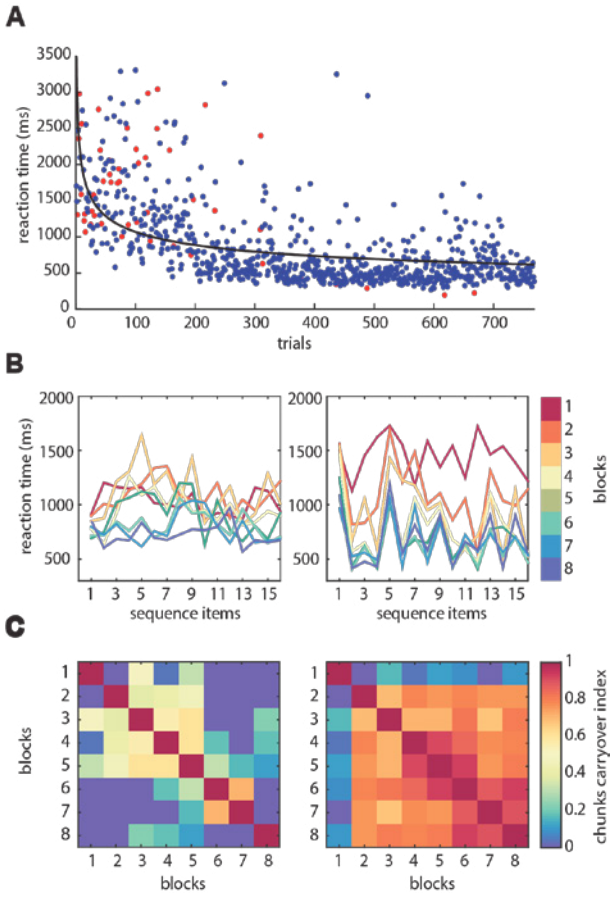
Despite the omnipresence of chunking - the grouping of sequence elements in smaller clusters - in sequence processing, its functional role remains unclear. Previous studies have failed to demonstrate any relationship between chunking and learning rate in motor sequence learning. Since motor control theories claim that movement optimization aims at minimizing energy cost, we predicted that chunking during motor sequences would help saving energy rather than help learning. Nineteen healthy subjects learned a motor sequence task using KINARM robotic device. We evaluated chunking by means of a novel algorithm and investigated its effect on several movement kinematics parameters. We found that participants who were better in chunking (i.e. relied on consistent chunking strategies) improved the most in terms of onset time and the kinetic energy. In addition, motor actions that were grouped into chunks exhibited shorter onset times and prolonged movement times, evoking a cost saving kinematic strategy. These findings suggest that chunking is involved in optimizing energy efficiency during motor sequence learning.



A. Movement trajectory during one representative trial. The Euclidean distance between the target position and the point in movement trajectory that was closest to the target denoted the spatial error (se). It was later normalized by subtracting, for every target, subject's average spatial error for that target during counterclockwise blocks; **B.** The distance from the central point (upper bar), and the distance towards the target (lower bar) were used to derive the following movement measures: onset time (ot) - the time interval between target appearance and a subject leaving 1 cm radius zone around the central point; movement time (mt) – the time interval between the robot handle leaving 1 cm radius zone around the central point and reaching 1 cm radius zone around the target

2.2. Chunking improves symbolic sequence processing and relies on working memory gating mechanisms

Chunking is ubiquitous during sequence processing. In addition to increasing WM capacity, efficient chunking strategy, by hypothetically decreasing WM load and freeing more cognitive resources also benefit sequence processing. However, so far, the impact of chunking on sequence learning performance strikingly lacks experimental support, since most measures of chunking have failed to correlate with indexes of performance or learning rate. Besides, the nature of the interactions between chunking and WM mechanisms during sequence learning remains debated. Computational models have posited that chunking relies on WM gating, i.e. the process that controls the access of new information to WM, but experimental evidence is still lacking. Here, we correlated individual estimates of chunking strategy in a sequence learning task with general task performance, cognitive workload (as indexed by pupil size) and performance in a WM updating task. We found that participants who adopted a consistent chunking strategy during symbolic sequence learning showed a greater improvement of their performance and a larger decrease in cognitive workload over time. Stronger reliance on chunking was also associated with higher scores in a WM updating task, suggesting the contribution of WM gating mechanisms to sequence chunking. Altogether, these results indicate that chunking is a cost saving strategy that enhances effectiveness of symbolic sequence learning.



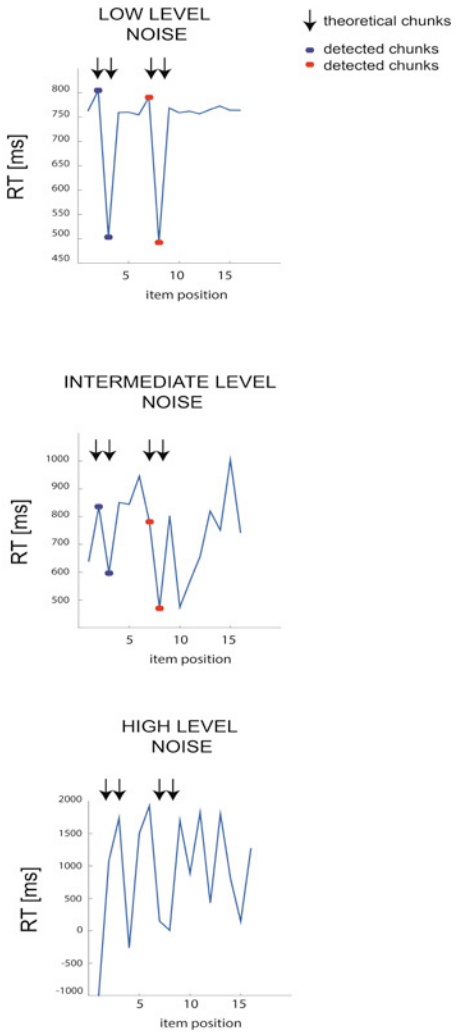
A. Reaction times from one example participant in the sequence-learning task. Trials in which an incorrect answer was given are marked in red. **B.** Reaction times of 2 example subjects averaged for every block of the sequence-learning task. **C.** Color matrix representing the coefficients of correlation between each pair of block-wise averaged reaction times of the two example subjects. The average of these coefficients provides the chunking carry-over index (Ci), used as a measure of chunking in the present study. Stochastic RT patterns lead to low Ci (e.g. left part of the figure) whereas systematic RT patterns, regarded as signatures of chunking, lead to high Ci (right part of the figure).

2.3. New ranking-based algorithm to determine chunking strategy

Chunking is a well-known mechanism used to lessen working memory load and it leads to measurable behavioral effects in terms of reaction times. Despite the fact that the topic has already been extensively studied, methods to identify and analyze chunking strategy remain discordant and difficult to implement. In this project, we propose a simple and reliable method to identify the number of chunks in a sequence and to determine their position and consistency.

The algorithm is based on a ranking approach that provides the position of the chunks, combined with an index that quantifies the consistency of the chunking pattern across sequence repetitions. We validated our algorithm on ad hoc simulated data and quantified its accuracy by varying the noise level in these data. Furthermore, we applied this algorithm to actual reaction time series gathered from 3 different experiments previously performed in the lab.

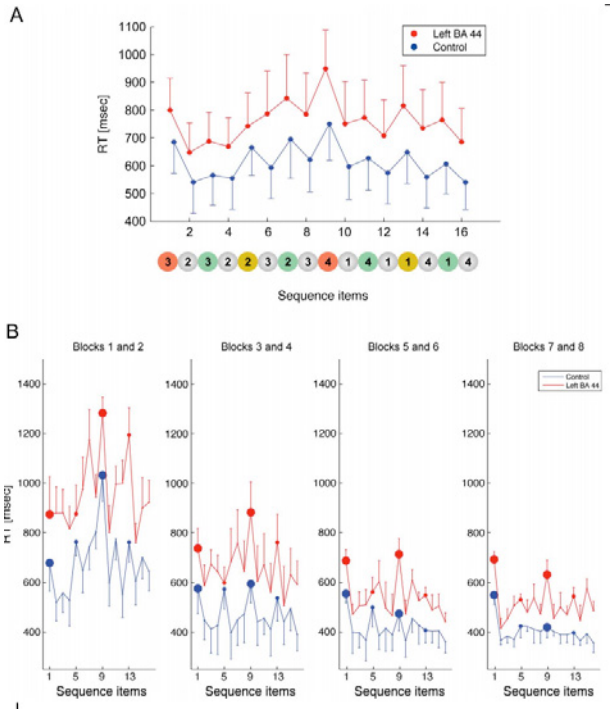
Surprisingly, our analysis showed that the chunking strategy adopted by the subjects remained constant after only a few blocks in all 3 experiments, a finding in contradiction with the classical view that, following an initial segmentation process, chunk concatenation occurs over time. Moreover, our results suggest a correlation between the consistency of the chunking strategy followed by the participants and their general learning performance.



Example of RT data for different noise levels. The arrows point to the theoretical chunks, while the red and blue dots show the chunks detected by our algorithm. Our algorithm was accurate except when the noise level was high.

2.4. Disruption of Broca's Area Alters Higher-order Chunking Processing during Perceptual Sequence Learning

Because Broca's area is known to be involved in many cognitive functions, including language, music, and action processing, several attempts have been made to propose a unifying theory of its role that emphasizes a possible contribution to syntactic processing. Recently, we have postulated that Broca's area might be involved in higher-order chunk processing during implicit learning of a motor sequence. Chunking is an information-processing mechanism that consists of grouping consecutive items in a sequence and is likely to be involved in all of the aforementioned cognitive processes. Demonstrating a contribution of Broca's area to chunking during the learning of a nonmotor sequence that does not involve language could shed new light on its function. To address this issue, we used offline MRI-guided TMS in healthy volunteers to disrupt the activity of either the posterior part of Broca's area (left Brodmann's area [BA] 44) or a control site just before participants learned a perceptual sequence structured in distinct hierarchical levels. We found that disruption of the left BA 44 increased the processing time of stimuli representing the boundaries of higher-order chunks and modified the chunking strategy. The current results highlight the possible role of the left BA 44 in building up effector-independent representations of higher-order events in structured sequences. This might clarify the contribution of Broca's area in processing hierarchical structures, a key mechanism in many cognitive functions, such as language and composite actions.

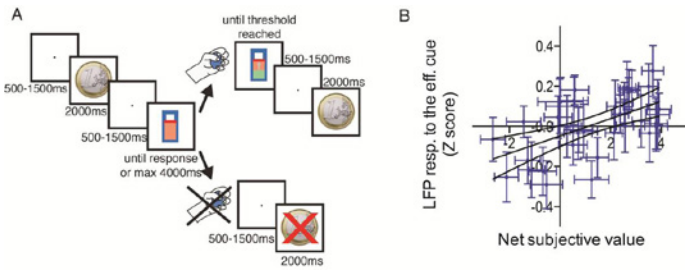


Chunking pattern. (A) RT for each sequence item (from 1 to 16) for all correct sequences in the control (blue lines) and left BA 44 (red lines) groups. Error bars indicate SE. (B) RT for each sequence item, as in A, but showing averaged data over pairs of blocks. Large and small dots represent Level 1 and Level 2 items of the sequence, respectively.

3. Contribution to the study of effort-based decision making and its link with dopamine

3.1. The Human Subthalamic Nucleus encodes the subjective value of reward and the cost of effort during decision-making

Adaptive behaviour entails the capacity to select actions as a function of their energy cost and expected value and the disruption of this faculty is now viewed as a possible cause of the symptoms of Parkinson disease (PD). Indirect evidence points to the involvement of the Subthalamic Nucleus (STN) - the most common target for deep brain stimulation (DBS) in PD - in cost-benefit computation. However, this putative function appears at odds with the current view that this nucleus is important for adjusting behaviour to conflict. Here we tested these contrasting hypotheses by recording the neuronal activity of the STN of patients with PD during an effort-based decision task. Local field potentials (LFP) were recorded from the STN of 12 advanced PD patients (mean age 63.8 years ± 6.8; mean disease duration 9.4 years ± 2.5) both OFF and ON levodopa while they had to decide whether to engage in an effort task based on the level of effort required and the value of the reward promised in return. The data were analysed using generalized linear mixed models and cluster-based permutation methods.



A. Depiction of the task used in the STN recording study. Patients were seeing first a reward, then an effort cue, indicating how much reward would be gained and how much effort would have to be executed if the offer was accepted. Then they could either start squeezing the dynamometer and maintain the effort up to a threshold indicated on a feedback gauge on the screen, or do nothing and skip to the next offer. **B.** The local field responses (LFP) to the effort cue (y-axis) were proportional to the net subjective value of the offer (x-axis).

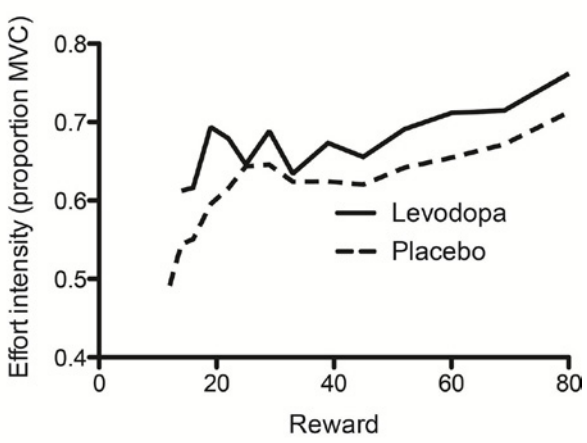
Behaviourally, the probability of trial acceptance increased with the reward value and decreased with the required effort level. Dopamine replacement therapy increased the rate of acceptance for efforts associated with low rewards. When recording the STN activity, we found a clear LFP response to both reward and effort cues in the 1-10 Hz range. In addition these responses were informative of the subjective value of reward and level of effort rather than their actual quantities, such that they were predictive of the participant's decisions. OFF levodopa, this link with acceptance was weakened. Finally, we found that these responses did not index conflict, since they did not vary as a function of the distance from indifference in the acceptance decision.

These findings show that low-frequency neuronal activity in the STN may encode the information required to make cost-benefit comparisons, rather than signal conflict. The link between these LFP responses and behaviour was stronger under dopamine replacement therapy. Our findings suggest that the STN mediates cost-benefit information that can then be used to decide whether or not to engage in effortful behaviour, and are consistent with the view that PD symptoms may be caused by a disruption of the processes involved in balancing the value of actions with their associated effort cost.

3.2. Dopamine manipulation affects response vigor independently of opportunity cost

Dopamine is known to be involved in regulating effort investment in relation to reward, and the disruption of this mechanism is thought to be central in some pathological situations such as Parkinson's disease, addiction and depression. According to an influential model, dopamine plays this role by encoding the opportunity cost, i.e. the average value of forfeited actions, which is an important parameter to take into account when making decisions about which action to undertake, and how fast to execute it. We tested this hypothesis by asking healthy participants to perform two effort-based decision-making tasks, following either placebo or levodopa intake in a double blind within-subject protocol. In the effort-constrained task, there was a trade-off between the amount of force exerted and the time spent

in executing the task, such that investing more effort decreased the opportunity cost. In the time-constrained task, the effort duration was constant but exerting more force allowed the subject to earn more substantial reward instead of saving time. Contrary to the model predictions, we found that levodopa caused an increase in the force exerted only in the time-constrained task, in which there was no trade-off between effort and opportunity cost. These findings were confirmed by the results of a computational model showing that dopamine manipulation left the opportunity cost estimate unaffected but altered the ratio between the effort cost and reinforcement value. These findings suggest that dopamine does not represent the opportunity cost but rather modulates how much effort a given reward is worth.

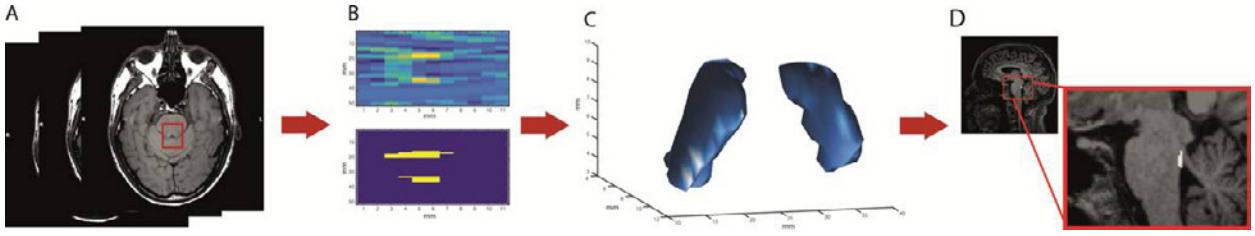


Levodopa intake (solid line) led to larger effort intensity (y-axis) than the control condition (dashed), irrespective of the reward level (x-axis).

4. Contribution to the study of mental fatigue in Parkinson's disease

4.1. Degeneration of locus coeruleus in Parkinson's disease covaries with fatigue.

Fatigue is one of the most incapacitating symptoms in Parkinson's disease (PD) but bears little relation to the disruption of the dopaminergic system: it evolves independently from the motor symptoms and is poorly treated by dopaminergic replacement therapy. Besides the Substantia Nigra, the Locus Coeruleus (LC), the main source of cortical noradrenaline, is also affected by the disease. We hypothesized that rather than the nigrostriate pathway, LC degeneration and the consequential disruption of the autonomic system would be directly responsible for PD fatigue. PD patients were asked to perform a psychomotor vigilance task (PVT) while different psychophysiological markers were measured (heart rate, respiratory rate, pupil size). Their level of fatigue, the quality of their sleep, and their degree of depression were evaluated through questionnaires (PFS, PDSS-2, BDI). Finally, we evaluated their degree of LC degeneration with a novel, automated method allowing to isolate the LC from neuromelanin MR imaging. Preliminary results (n=20) showed that LC degeneration correlated with the fatigue scores (coeff.=-0.51, p=0.02) but not with the degree of depression or the quality of sleep. Surprisingly, we found that LC degeneration failed to correlate with any of the psychophysiological measures of autonomic responses during the PVT. These findings suggest that LC degeneration may be responsible for fatigue in PD. They also suggest that the increase in heart rate, respiratory rate and changes in pupil size induced by target detection during the PVT may not depend primarily on the LC.



A. A volume of interest (VOI) was extracted from the neuromelanin-sensitive anatomical images, in accordance with our prior knowledge of the theoretical LC location and size (a 12 mm high cube with frenulum as a superior limit, and height/width of 60/50 mm, with the fourth ventricle as a dorsal boundary. **B.** All slices were visually inspected and the slice with the peak signal intensity was selected as a reference slice. In order to identify the voxels belonging to LC, k-means (k=3) algorithm was applied to every slice of the VOI. Thus, every voxel was assigned to one of the three classes: zero intensity, noise or LC. **C.** A smoothed 3D model of LC identified in this particular patient, shown for visualization purposes. **D.** LC superimposed in blue on whole brain anatomical images.

5. Publications.

5.1. Full papers

- Alamia A., Solopchuk O., d'Ausilio A., Van Bever V., Fadiga L., Olivier E., Zénon A. (In Press) Disruption of Broca's Area Alters Higher-order Chunking Processing during Perceptual Sequence Learning. **Journal of Cognitive Neuroscience** (see Annex).
- Alamia A., Solopchuk O., Olivier E., Zénon A. New ranking-based algorithm to determine chunking strategy. *Submitted.*
- Solopchuk O., Alamia A., Lefèvre P., Olivier E., Zénon A., Orban de Xivry J.J. Chunking is a cost saving strategy in motor sequence learning. *Submitted.*
- Solopchuk O., Alamia A., Olivier E., Zénon (In Press) A. Chunking improves symbolic sequence processing and relies on working memory gating mechanisms. **Learning and memory** (see Annex).
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Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
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The ephrin axon repellent system in amyotrophic lateral sclerosis

1. 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.

2. Results

2.1. WP1. Mechanism of involvement of EphA4 in motor neuron degeneration

WP1.1. 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 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 is deleted in all cell types. At the moment the triple transgenic mice Thy1-CreER::EphA4 $^{lox/lox}$::SOD1^{G93A} and CAGG-CreER::EphA4 $^{lox/lox}$::SOD1^{G93A} have been obtained and the first mice are being treated with tamoxifen at day 60 and followed to study disease parameters.
We have determined the efficiency of EphA4 excision in CAGG-CreER::EphA4$^{lox/lox}$ and CAGG-CreER::EphA4$^{lox/lox}$ after tamoxifen administration and compared it to EphA4$^{lox/lox}$ mice. Mice were administered with 4 mg tamoxifen at 3 months of age during 4 consecutive days, and were sacrificed 14 days after the last tamoxifen administration. CAGG-CreER::EphA4$^{lox/lox}$ mice presented a reduction of 85% of EphA4 mRNA and protein levels, whereas Thy1-CreER::EphA4$^{lox/lox}$ mice showed a reduction of about 30% of EphA4 mRNA and protein levels as compared to EphA4$^{lox/lox}$ mice, consistent with the fact that in the later mouse EphA4 is only excised in neurons (Figure 1).

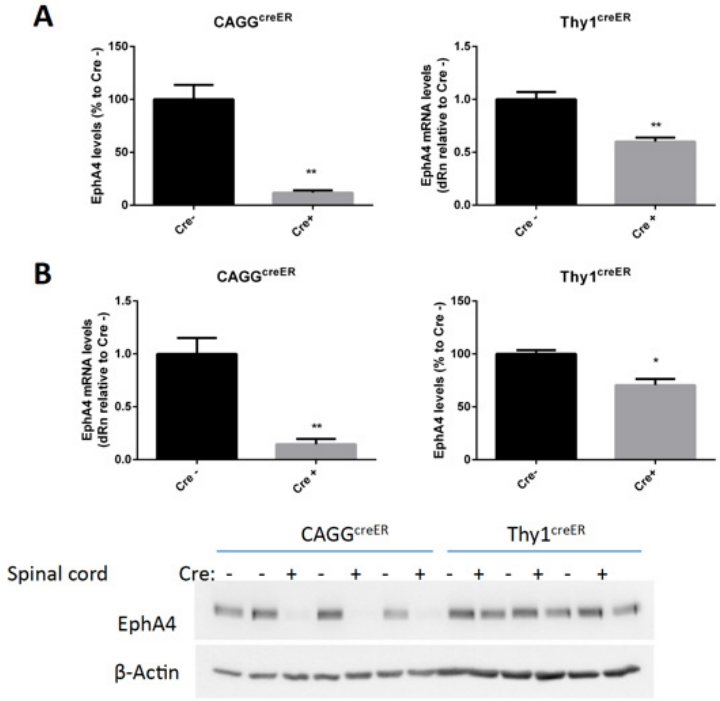

Figure 1. CAGG-CreER::EphA4$^{lox/lox}$ and Thy1-CreER::EphA4$^{lox/lox}$ mice have reduced levels of EphA4 mRNA and protein after tamoxifen administration. EphA4 mRNA (A) and protein (B) levels were quantified in the spinal cord of 3-months-old mice treated with tamoxifen by qPCR and western-blot respectively.

WP1.2. 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, as determined with the hanging wire test, nor disease survival is altered in EphA4-eGFP$^{+/-}$::SOD1^{G93A} mice compared to SOD1^{G93A} mice (Figure 2A, 2B). Neither the percentage of innervated neuromuscular junctions (NMJs) differed in EphA4-eGFP$^{+/-}$::SOD1^{G93A} compared to SOD1^{G93A} symptomatic mice (Figure 2C). We are currently quantifying the number of motor neurons in the spinal cord of EphA4-eGFP$^{+/-}$::SOD1^{G93A} and SOD1^{G93A} mice.

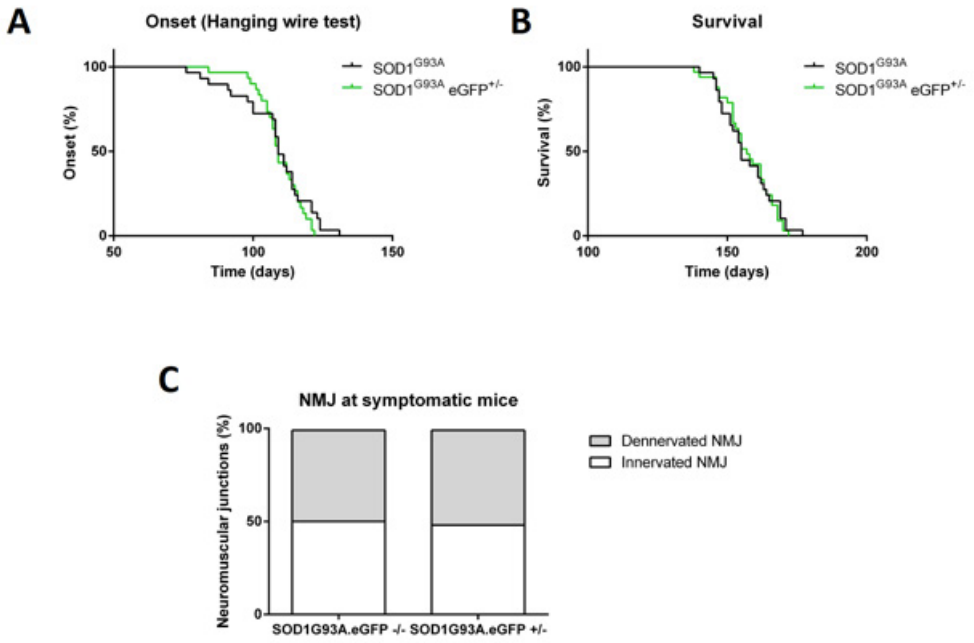

Figure 2. Forward signalling does not play a role in ALS as determined with the EphA4^{eGFP}mouse. Onset (A), which was determined by lack of performance on the hanging wire test (N=27-30), and survival (B) were determined in EphA4^{eGFP}::SOD1^{G93A} and compared to SOD1^{G93A} mice (N=29-30). No difference 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}). (C) Percentage of innervated and dennervated neuromuscular junctions were assessed in both SOD1^{G93A} and SOD1^{G93A} EphA4eGFP$^{+/-}$ mice, but no differences were found (49% innervated NMJ in EphA4^{eGFP}::SOD1^{G93A} compared to 51% innervated NMJ in SOD1^{G93A}).

These results might indicate that forward signalling is not playing a role in ALS. However, 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].

WP1.3. 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. In preliminary experiments, we 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 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) and generated a triple transgenic mouse to specifically excise ephrin-B2 from astrocytes in SOD1^{G93A} mice, the Cx30-CreER::ephrin-B2$^{flox/flox}$::SOD1^{G93A}. Disease parameters have been studied in these mice after tamoxifen treatment at 60 days of age, and have been compared to ephrin-B2$^{flox/flox}$::SOD1^{G93A} mice also administered with tamoxifen. No difference was observed in onset, but survival was significantly worsened by 9 days (Figure 3).

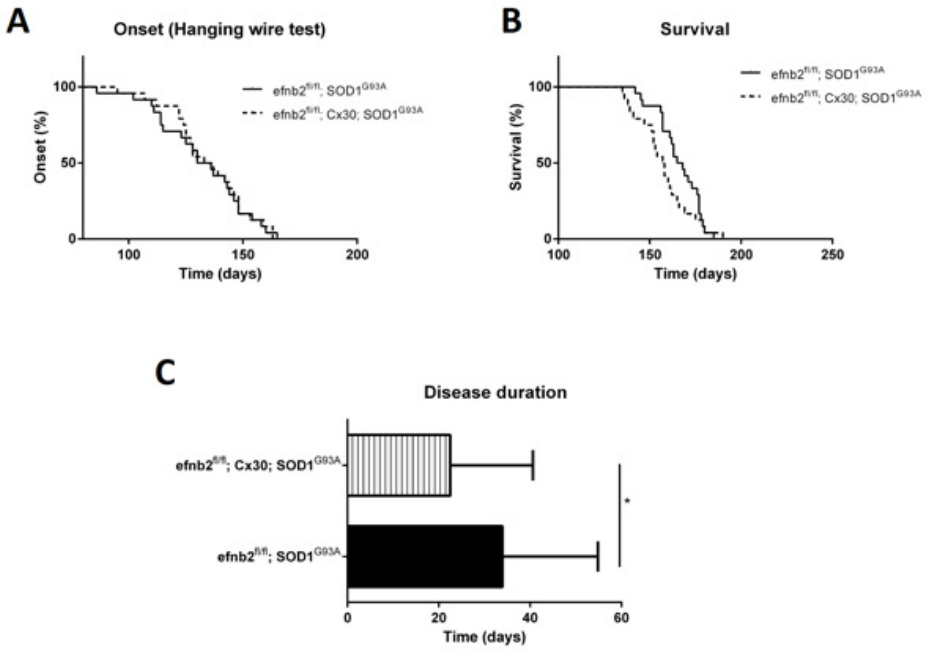

Figure 3. Excision of ephrin-B2 from astrocytes from the age of 60 days on does not alter disease onset and worsens survival. (A) Onset analysis, as measured with the hanging wire test, does not show any significant delay: 135 days (Cx30-CreER::ephrin-B2$^{flox/flox}$::SOD1^{G93A}, n = 24) versus 133 days (ephrin-B2$^{flox/flox}$::SOD1^{G93A}, n = 24). (B) Survival is decreased in these mice (157 days, Cx30-CreER::ephrin-B2$^{flox/flox}$::SOD1^{G93A}, n = 24; versus 166 days, ephrin-B2$^{flox/flox}$::SOD1^{G93A}, n = 24; p = 0.0297) and (C) disease duration is also shorter in mice lacking ephrin-B2 (23 days, Cx30-CreER::ephrin-B2$^{flox/flox}$::SOD1^{G93A}, n = 24; versus 34 days, ephrin-B2$^{flox/flox}$::SOD1^{G93A}, n = 24; p = 0.0482).

Based on our current findings deletion of ephrin-B2 from the astrocytes does not seem to be beneficial in a mouse model of ALS. Although astrocytic ephrin-B2 does not play a similar beneficial role as EphA4 in ALS, and thus does not seem to participate in the same mechanism of action, a deeper analysis of the function of this ephrin ligand in disease is important and will be carried out in the future.

2.2. WP2. Therapeutic significance of the involvement of EphA4 in motor neuron degeneration

WP2.1. Use of KYL, an EphA4 antagonist

In a previous report we mentioned that we 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. Since at a dose of 10 mM the motor performance of mice on the rotarod was slightly (and statistically non-significantly) affected, and at a concentration of 5 mM there was a risk of drug precipitation inside the pumps, we chose to perform these experiments with KYL peptide at a concentration to 3 mM and 1.5 mM. During the last months, we have administered the peptide intracerebroventricularly to cohorts of 60 days-old SOD1^{G93A} mice, corresponding to a presymptomatic stage of the disease, until they were end-stage using Alzet miniosmotic pumps. As controls we have injected not only vehicle (ACSF) but also the peptide with a tyrosine-alanine substitution that completely abolishes the inhibitory function (KAL peptide). We evaluated the 4 groups using clinical parameters: disease onset and survival. Unfortunately, no delay in onset or extended survival was found (Figure 4).

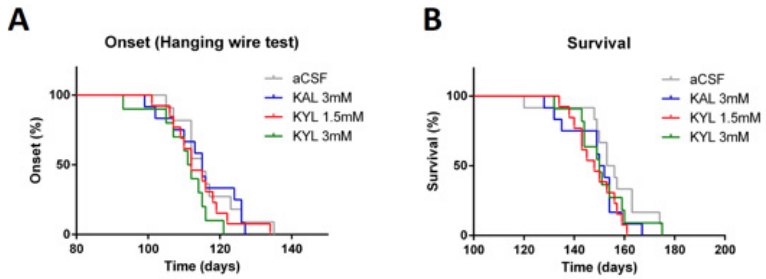

Figure 4. Treatment with KYL peptide does not improve disease parameters in the SOD1^{G93A} mouse model of ALS. Different concentrations of KYL peptide were infused intracerebroventricularly in SOD1^{G93A} mice with Alzet miniosmotic pumps. ACSF and KAL were infused as controls. No significance difference was observed on onset (median onset was: ACSF, 115 days; KAL 3mM, 115 days; KYL 3 mM, 112 days and KYL 1.5 mM, 112 days) and neither on survival (median survival was: ACSF, 155 days; KAL 3mM, 151 days; KYL 3 mM, 150 days and KYL 1.5 mM, 148 days) N=10-13.

WP2.2. 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 (Nb) 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 15 Nb, single-chain antibodies produced in *Camelidae*, directed against the extracellular domain of EphA4, using previously described methods [9]. From the generated Nb, 5 Nb resulted in low expression levels in a bacterial system and presented tendency to aggregate. Therefore these 5 Nb were excluded from the screen. As mentioned in a previous report, affinity for EphA4 was thereafter determined for the 10 Nb left by using surface plasmon resonance (SPR, Biacore). The EphA4 ligand-binding domain (LBD) was immobilized on a chip and Nbs with concentrations from 1 nM to 300 nM were used as analyte. SPR analysis revealed that whereas Nb 34, Nb 47 and Nb 19 only showed none or low binding affinity, the other seven Nbs show affinity in low nanomolar range. For those nanobodies with the best K_d, the specificity for EphA4 binding was determined next by analyzing the affinity for other Eph receptors by using SPR. SPR analysis revealed that Nbs 50, 57 and 60 had the highest non-specific binding to Eph receptors, so we excluded them from further screening (Figure 5). However, Nbs 22, 39, 31 and 53 were the most selective for EphA4 with some binding also to the EphA7 receptor (Figure 5).

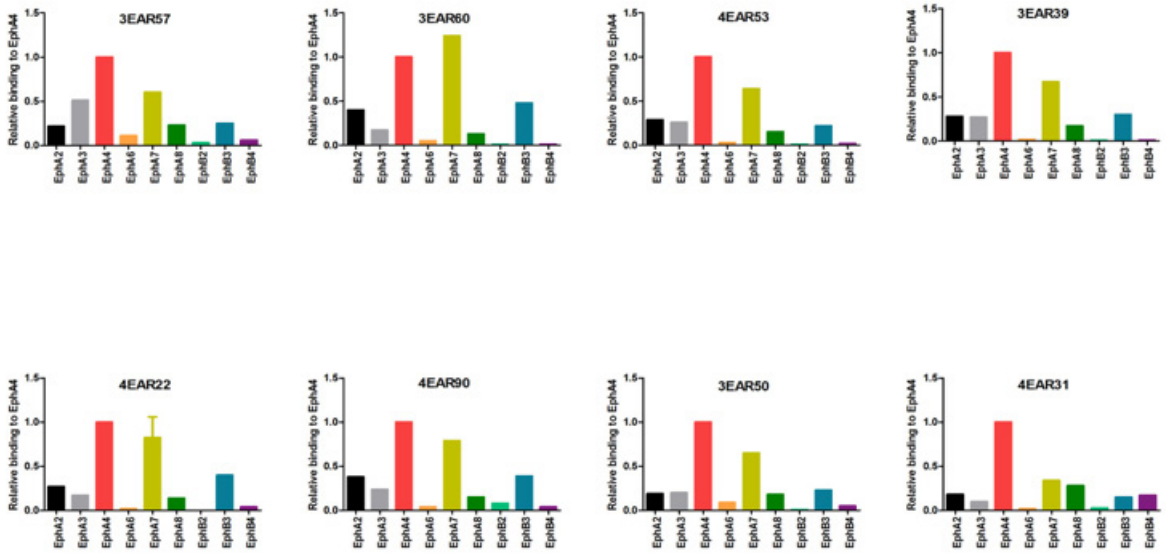

Figure 5. Screening for the EphA4 nanobody with the best specificity for EphA4. Binding affinity results of the different nanobodies to different Eph receptors are presented, as determined with SPR (Biacore).

In order to define the binding properties of the Nbs to the EphA4 such as their specific binding site in the LBD domain and the biological significance of such binding, we next tested the Nbs on binding competition assays with EphA4 ligands as well as activity assays of the EphA4 receptor. In all experiments we used the KYL peptide as a positive control, and also to determine if our aim in obtaining Nbs that would work better than the KYL peptide would be accomplished. Nb 22 could almost completely inhibit the binding of ephrin-A3 and ephrin-B ligands, but not others, to EphA4. Nb 31 completely inhibited the binding of ephrin-A1, A3, B1, B2 at equal or even at lower concentrations than the KYL-peptide. Nb 39 and Nb 53 reached complete inhibition of ligand binding for all ephrin ligands in a concentration range lower than the KYL-peptide. Thus, these two Nbs seem to be the most interesting outcome of our study (Figure 6).

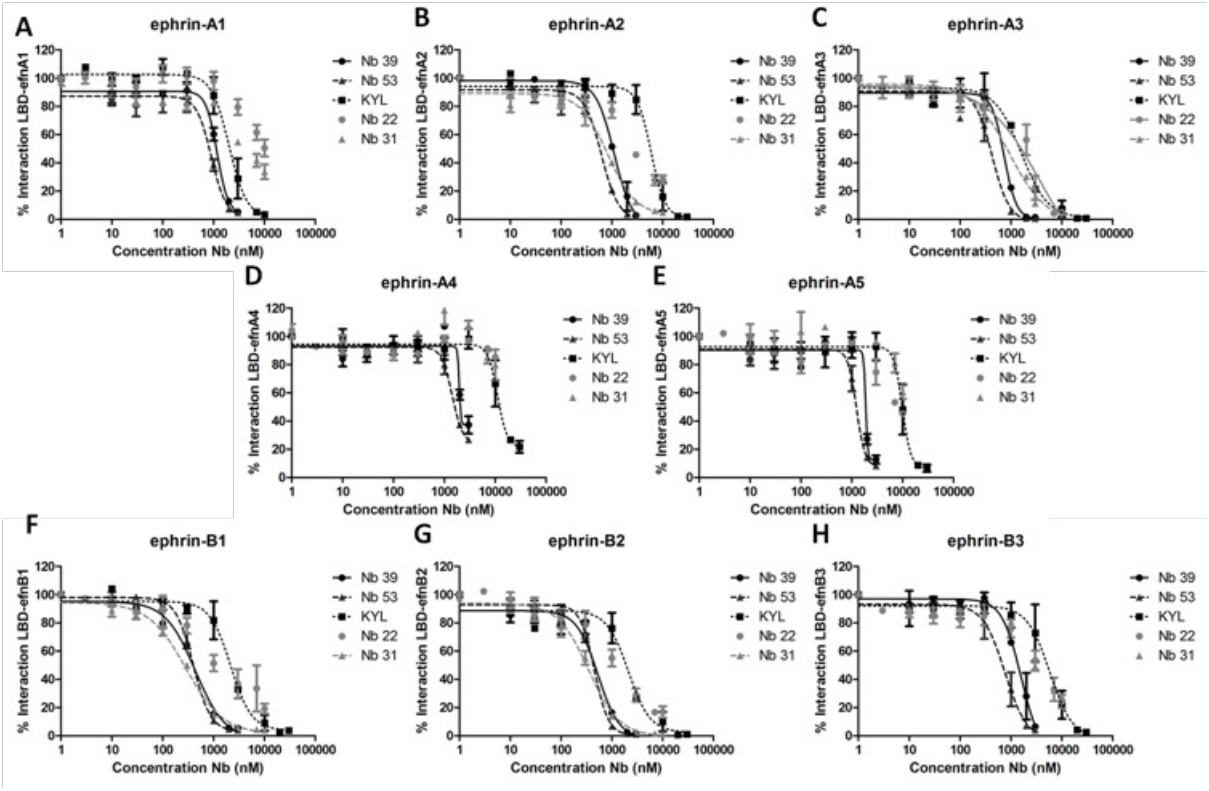

Figure 6. Nb 39 and 53 bind EphA4 and strongly compete with the binding of all EphA4 ligands. Increasing concentrations of Nb were assessed to determine the capability of these molecules to inhibit the binding of ephrin ligands to the EphA4 receptor. The KYL peptide is always used as a positive control. Values represented indicate mean with SD.

To assess the antagonistic properties of the Nbs we used the DiscoverX PathHunter U2OS EphA4 cell line (DiscoverX) in which ephrin-induced activation of EphA4 in genetically modified cells causes activation and dimerization of the receptor leading to crossphosphorylation and yielding to an active β-galactisodase enzyme, which can be visualized. Increasing concentrations of the Nbs were added to the medium before ephrin-A1 stimulation. Nb 31 only reached 40% inhibition with the concentrations tested, whereas Nb 39, 53 and 22 reached complete inhibition of ephrin-A1 induced phosphorylation at lower concentrations compared to the KYL-peptide (Figure 7). Remarkably, Nb 22 yielded similar inhibition as Nb 39 and Nb 53 although it did not inhibit the interaction with ephrin-A1 to a similar extent (Figure 6).

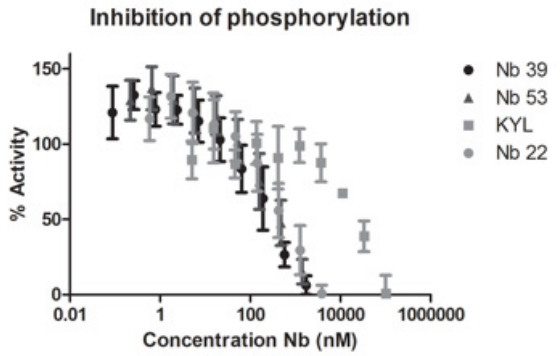

Figure 7. Nb39, 53 and 22 antagonize the activation of EphA4 upon ligand stimulation at a higher efficiency than the KYL peptide. Increasing concentrations of Nb were assessed to determine the capability of these molecules to inhibit the phosphorylation and activation of the EphA4 receptor after stimulation with ephrin-A1 ligand. The KYL peptide is always used as a positive control. Values represented indicate mean with SD.

In order to have a functional readout of the inhibitory capacity of nanobodies, we are assessing them in a growth cone collapse assay. E13.5 motor neuron primary cultures are incubated with Nb prior to stimulation with an EphA4 ligand, which induces growth cone collapse and actin filaments retraction at the end of the neurites. The percentage of collapse at each culture is being measured (Figure 8). As soon as we have the results of this experiment, we will be able to select the best Nb, which will be used for a clinical trial in mice.

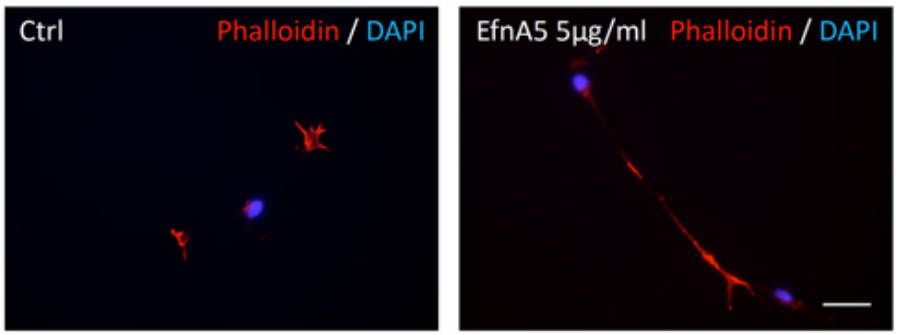

Figure 8. Optimization of ephrin-A5 induced growth cone collapse. Motor neuron cultures performed at E13.5 were cultured for 24 hours and treated with 5µg/ml ephrin-A5-Fc recombinant protein (EfnA5) or Fc recombinant protein (Ctrl) for 30 minutes. In order to visualize actin filaments and growth cones, cultures were stained with phalloidin-Alexa555 and DAPI.

WP2.3. 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 subarachnoid haemorrhage, 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 9).

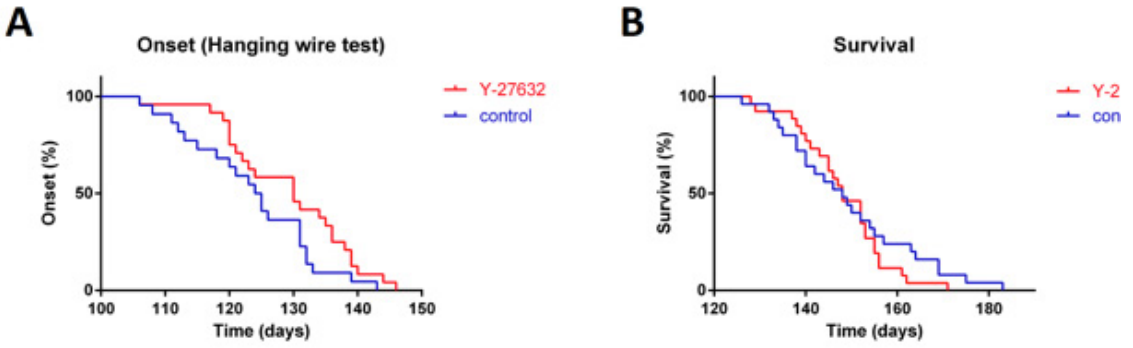

Figure 9. Treatment of SOD1^{G93A} mice with Y-27362 did not improve onset or survival. Onset analysis of control (124.5 days, n=22) and Y-27362 (130 days, n=24) treatment. Survival analysis of control (148 days, n=25) and Y-27362 (148 days, n=26) treatment.

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 on survival (Figure 10).

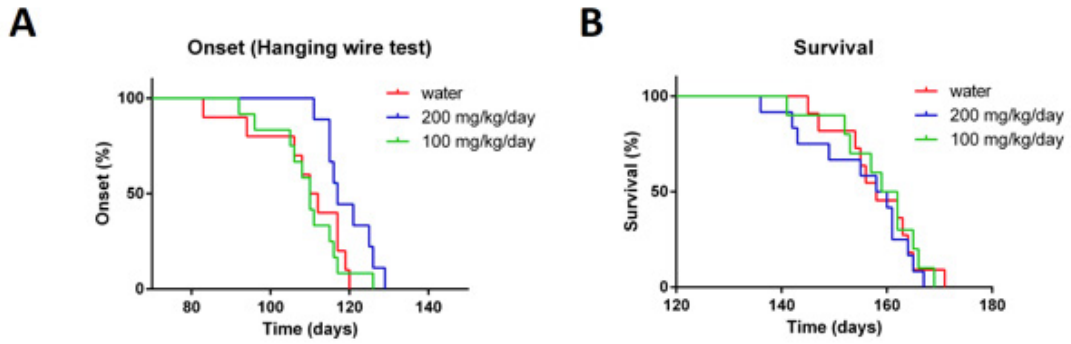

Figure 10. Treatment of $SOD1^{G93A}$ mice with fasudil delayed onset, but did not affect survival. Onset analysis of control (111 days, n=11), 100 mg/kg/day (110 days, n=12) and 200 (117 days, n=9) mg/kg/day fasudil. Survival analysis of control (158 days, n=11), 100 (160.5 days, n=12) and 200 (159 days, n=9) treatment.

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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 2015 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.

On last year, we summarized the setting up of optogenetics both by using activating, the light-sensitive cation channel Channelrhododopsin-2 (ChR2), and silencing, the proton pump Archaerhodopsin-3 (ArCh), opsins to either activate or silence neurons, respectively. Ex vivo and in vivo functional experiments validated the approach and the selectivity of expression ands consequences in D1R- and D2R-neurons. The in vivo technique allow to deliver optical stimulation deep into the brain area of interest in freely moving animals and we have now used it to study the behavioral outcomes of light-induced neuronal activity from the different striatal subpopulations in different learning tasks.

The pharmacogenetic approach called Designer Receptors Exclusively Activated by Designer Drugs (DREADD) is another alternative to cell-specific ablation and was developed in the lab. 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. We applied this technique in Adora2a-Cre mice to selectively target the indirect pathway through Adora2a/D2R-expressing striatopallidal neurons. We first validated the specific expression of the hM4Di transgene in striatopallidal neurons exhibiting almost a full-striatum diffusion (Figure 1A-C). We quantified that 98.9 ± 0.6% of hM4Di-mCherry-expressing neurons are Adora2a positive, and that we transfected 56.8 ± 4.6% of Adora2a neurons with the hM4Di-mCherry transgene in coronal sections of the central striatum (Figure 1B,C). We then tested the effect of CNO injection on mice locomotor behaviour (Figure 1D). The pharmacogenetic inactivation of Adora2a/D2R neurons induces a progressive increase in locomotion measured as the distance travelled (Figure 1D) and enhanced velocity (not shown) in a new open field exploration test. We are now examining the consequences of such hyperactive behaviour.

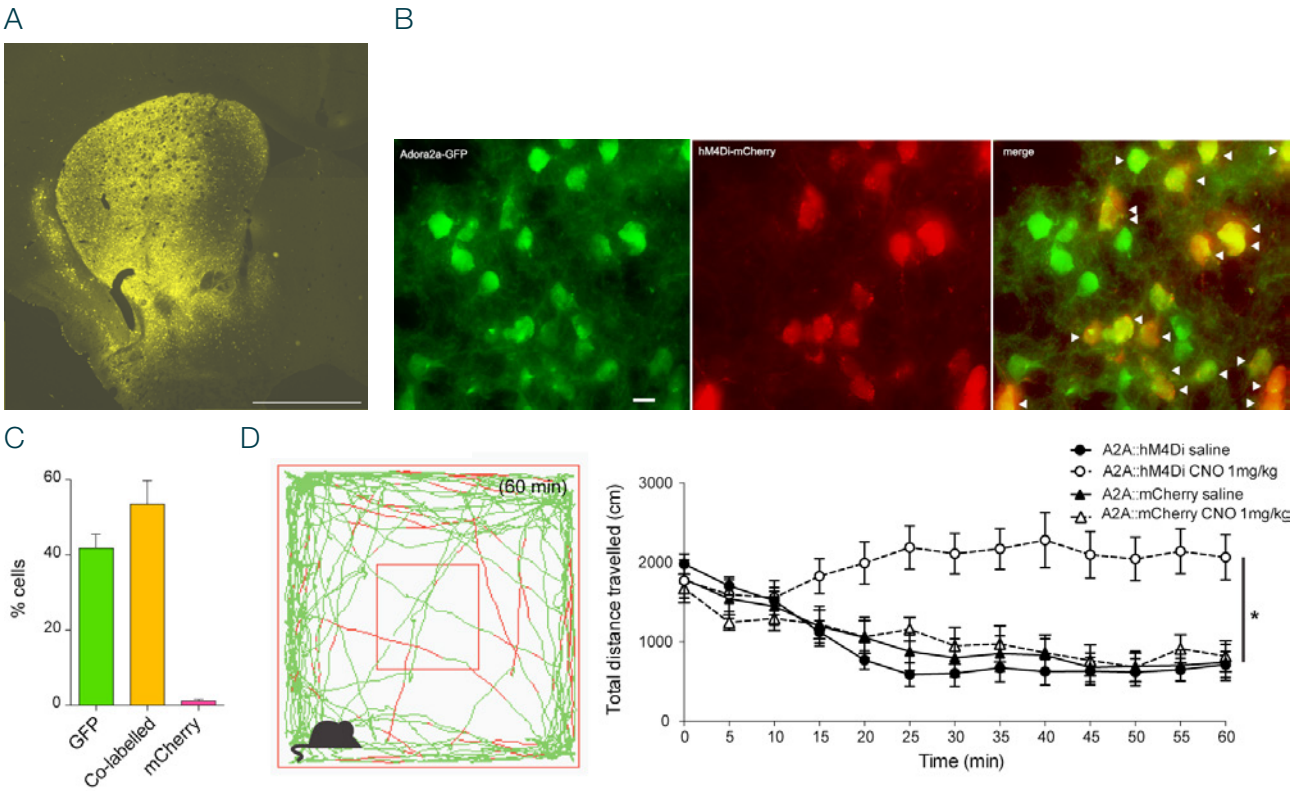

Figure 1: **Functional validation of pharmacogenetic (DREADD) inactivation of striatopallidal neurons.** A) Photomicrograph depicting hM4Di-mCherry transgene expression in the right hemisphere striatum of an Adora2a-Cre mouse. Scale bar: 1mm. B) Photomicrograph showing the selective targeting of hm4Di-mCherry (red) expression in Adora2a-GFP neurons in a central section of the striatum. White arrows in the merge picture indicate Adora2a-GFP and hM4Di-mCherry co-labelled cells. Scale bar: 10um. C) Histograms representing the cell count of Adora2a-GFP single-labelled cells (green), Adora2a-GFP/hM4Di-mCherry co-labelled cells (orange) and very few hM4Di-mCherry single-labelled cells (red). Data are represented as mean ± SD. D) Schematic of the new open field exploration test in which the mice behaviour are videotracked to measure distance travelled and velocity. Total distance travelled is acquired in bins of 5min during a 1h session test. Mice in which Adora2a-Cre neurons are transfected with the hM4Di-mCherry transgene both receive randomly saline (black circles) and CNO (1mg/kg: white circles) through intraperitoneal injections and are immediately placed in the open field to track their locomotion (n=9). Control mice consist of Adora2a-Cre littermates transfected with the control AAV-mCherry virus (n=8; black triangles: saline and white triangles: CNO). Data are represented as mean ± SEM. * $p<0.05$ $F(2,34)=4.93$, 2-way RM ANOVA.

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., 2015). 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., 2015).

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. As stated on last year, for this, we developed and validated optogenetics for FSI in the striatum. We are now analyzing the network properties of these FSI *in vitro* in terms of their control of MSNs activity and evaluating the involvement of these neurons in striatal-dependent behaviors.

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. 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-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-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. This result indicates that iMSN NMDA-R deletion may change the process of action selection within the BG. To further characterize this behavioural alteration we performed a more sophisticated motor-skill learning paradigm: the single-pellet reaching task. This paradigm requires precise and coordinated motor movements of the forelimb, as mice reach for and retrieve objects through a narrow slit. The speed and accuracy of the successful movements were quantified across daily sessions. The average speed as well as the average success rate over time during the training period of the single-pellet reaching task were significantly higher for the control mice compared to cKO mice and the cKO mice were unable to learn the task. In view of 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-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-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-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-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-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-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-Cre$^{+/-}$ Grin1$^{f/f}$ and A_{2A}R-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. 3D reconstruction of the recorded neurons of the recorded neurons showed that NR1-deficient striatopallidal neurons displayed a decreased dendritic arborisation and a reduction in spine density. Consistent with these morphological alterations, spontaneous excitatory postsynaptic currents (sEPSCs) amplitudes were decreased demonstrating the dimming of the corticostriatal synaptic transmission. 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. 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.

All these results have been submitted for publication in a revised version (Lambot et al., 2015)

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 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 have used 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 has been 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 have partially identified the cellular factors involved in the signaling pathway, among which the kinase Src seems to play an important role.

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. We 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 and that this effect is specific to striatopallisal neurons.

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

- Ciliary transport is required for ciliogenesis, signal transduction and trafficking of receptors to the primary cilium. Mutations in inositol polyphosphate 5-phosphatase E (INPP5E) have been associated with ciliary dysfunction, however, its role in regulating ciliary PIPs is unknown. By using conditional inactivation and high resolution confocal microscopy, we have reported that in neural stem cells

(NSC), phosphatidylinositol 4-phosphate (PI4P) is found in high levels in cilia while phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P2] is not detectable. On INPP5E inactivation, PI(4,5)P2 accumulates at the ciliary tip while PI4P is depleted. This is accompanied by recruitment of the PI(4,5)P2 -interacting protein TULP3 to the ciliary membrane along with Gpr161. This results in an increased production of cAMP and a repression of Shh transcription gene Gli1. Our results have revealed for the first time, the link between ciliary regulation of PIPs by INPP5E and Shh regulation via ciliary trafficking of TULP3/Gpr161, and have provided a mechanistic insight into ciliary alterations found in Joubert and Morm syndromes resulting from INPP5E mutations (Chavez et al., 2015).

- 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., 2015).

6. Publications 2015

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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

Celsr (Cadherin, EGF-like, Laminin G-like, Seven-pass, G-type Receptor) are developmentally regulated proteins with the capability 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 behavior 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 developmental disorders.

When we started studying the PCP proteins Celsr, two members (Celsr1 and Celsr2) were listed in databases, but almost nothing was known about their expression or their function. We identified the third member (Celsr3), explored the expression patterns of the three Celsrs, and assessed their roles in the developing nervous system. We found that Celsr cadherins act as sorting factors for two PCP protein complexes that assemble at the membrane and govern distinct aspects of CNS development and function. The first complex, composed of Celsr1, Fzd3 and Vangl2, is mainly active in germinal zones, and is important in neural tube closure, neuronal migration along the rostrocaudal axis, and cilia polarity. The second, encompassing Celsr2, Celsr3 and Fzd3, is active in postmitotic cells and is critical, among others, in axon guidance and brain wiring (reviewed in Tissir et al. Eur J Neurosci 2006; Tissir et al. Curr Opin Neurobiol 2010, Boutin et al. Curr Top Dev Biol 2012, Tissir et al. Nat Rev Neurosci 2013, Chai et al. Int J Biochem Cell Biol 2015).

In 2015, we have progressed on the following topics:

1. PCP in the wiring of the nervous system

Since our seminal work involving the PCP in axon guidance (Tissir et al. Nat Neurosci 2005), we and others have accumulated evidence that PCP signaling is a major player in the wiring of the central nervous system. During the last year in a collaborative work, we analyzed and reported on the function of PCP in striatal connectivity. We find that, within the striatum, PCP genes are required *in vivo* for extension of medium spiny neurons (MSNs, the striatal output neurons forming prominent descending axon tracts). In addition, PCP proteins non–cell–autonomously regulate the entry of MSN axons into an important intermediate target, the globus pallidus (pallidum), and the positioning of “corridor” cells. These cells are guidepost cells for thalamocortical axons located in between the pallidum and the medial ganglionic eminence. We showed that corridor cells repel MSN axons and that their mislocalization in the GP of PCP mutant mice render the GP non–permissive for MSN axon growth. Conclusively, these data reveal previously uncharacterized aspects of striatal pathway development and identify cell autonomous and non–cell-autonomous roles for PCP in MSN axon guidance (Morello et al. J Neurosci 2015).

A major issue with the identification of PCP partners and downstream effectors in wiring of the nervous system has been the lack of suitable antibodies. We have been trying for more than 10 years to produce antibodies both in our lab and as a service from dedicated companies. Finally, we have succeeded very recently to produce valuable resources. In particular we have produced two antibodies (a mono and a polyclonal) that detect endogenous Celsr3 by western blotting in a highly specific manner as well as a polyclonal antibody against Fzd3. The anti–Celsr3 and anti–Fzd3 polyclonal antibodies could also precipitate the proteins from transfected cells as well as from brain tissue. In 2015, we have analyzed

Celsr3– and Fzd3–immunopecipitates by mass spectrometry and we are in the process of evaluating and validating the most promising interactors.

2. PCP genes in neurogenesis

During cortical development, apical progenitor cells (AP) undergo divisions that are initially symmetrical and increase the pool of progenitors. At the onset of neurogenesis, some divisions become asymmetric and generate, in addition to AP, either neurons or intermediate/basal progenitors (BP) which have limited self–renewal capacity and are committed to an excitatory glutamatergic neuron fate. In the dorsal telencephalic wall, neural progenitor cells (NPC, including AP and BP) generate sequentially deep layer (DL, mostly layers 5 and 6) and upper layer (UL, mostly layers 2, 3 and 4) neurons, followed by glial cells. An interesting question in the field is how those processes (i.e. type of division of progenitors, the neurogenic to gliogenic switch) are orchestrated at the molecular and cellular level.

Celsr1 is exclusively expressed by NPC; and little, if any, is known about it function therein. We examined the distribution of Celsr1 protein in the mouse neocortex and found that it was confined to the apical side of AP until embryonic day (e) 10.5. At the onset of neurogenesis (from e12.5 onwards), Celsr1 accumulated progressively in basal processes and endfeet of AP. Celsr1 being an adhesion protein, we investigated the attachment of AP processes to the pia in *Celsr1*$^{-/-}$ mice. AP were radially oriented and their endfeet were in close proximity with the basal lamina. We did not observe any breakdown in the latter or neuronal heterotopia that would suggest an abnormal contact between endfeet and the pial surface. We examined the morphology of APs upon *Gfp* electroporation and found that the number of basal processes was dramatically reduced in *Celsr1*$^{-/-}$ mice. Given the morphological change of AP in *Celsr1*$^{-/-}$ mice, we asked to what extend this modification could modify neurogenesis. We used a tamoxifen inducible system to label AP and trace their progeny. Compared to controls, mutant AP generated more cells in the ventricular zone (VZ) and less in the SVZ or intermediate zone, suggesting a defect of neurogenesis in *Celsr1*$^{-/-}$ mice. To assess the effect of *Celsr1* loss–of–function on fate decision, we injected mice carrying a floxed allele (Celsr1^{f}) with low concentration of Tamoxifen at e10.5, with the aim to inactivate *Celsr1* in isolated AP and enable a clonal analysis at e14.5. *Enface* view scrutiny of VZ detected larger clones in *Celsr1* conditional mutant embryos, confirming that *Celsr1*$^{-/-}$ AP are more prone to symmetric, proliferative division. We measured the length of the VZ and found that it was increased from e12.5 onwards in mutant versus control samples. At e16.5, the higher expansion of mutant AP led to local bending of the VZ with an increased number of Pax6-positive AP. The number of Tbr2–positive BP was concomitantly reduced, first in the lateral pallium at e12.5, and later in the dorsal pallium at e14.5. This bias in BP and thus in neuron production led to microcephaly with a reduction of cortical upper layers at postnatal stages.

We then inactivated *Celsr1* in all neocortical areas and analyzed the impact loss–of–function of Celsr1 on behavior. Cortex-specific conditional mice (*Celsr1*$^{f/f}$*; Emx1–Cre*) were all hyperactive and spent more time than controls (*Celsr1*$^{+/+}$*; Emx1–Cre*) in open areas in elevated plus maze and open field. In addition, females had a social interaction defect in the ‘‘three chamber test’’. Similar defects are found in rodent models of Autism Spectrum Disorder (ASD). Surprisingly, further to the abnormal behavior observed in the open field and the elevated plus maze, conditional knockout males had learning deficit in the water maze test. This phenotypic defects correlate with Attention-Deficit Hyperactivity Disorder (ADHD) in rodents; a trait with documented comorbidity with ASD. These results suggest that the loss of Celsr1 and subsequent reduction in the number of endfeet promote proliferative division of AP at the expense of neurogenic divisions in the developing neocortex, decreases the number of upper layer neurons, and ultimately perturb cognitive abilities functions.

Endfeet of APs abut meninges which regulate brain development through secreted molecules. For instance, retinoic acid (RA) derived from meninges triggers the switch from proliferative to neurogenic divisions at the onset of neurogenesis. Of note, meningeal cells that produce RA appear progressively around the telencephalon in a lateral to dorsal gradient between e12.5 and e14.5, which correlates with the defect in BP production seen in *Celsr1*$^{-/-}$ cortex. In addition, Crabp2, a cytosol-to-nuclear shuttling protein facilitating the binding of RA to its cognate nuclear receptors, accumulates in AP endfeet and could improve RA uptake and its relocation to the nucleus. To test whether RA is indeed involved in the defective neurogenesis observed in mutant mice, we exposed *Celsr1*$^{-/-}$ embryos to RA supplementation *in utero* between e11.5 and e14.5. This treatment restored the number of Tbr2–positive BP and the length of the VZ at e14.5, confirming the causal link between RA and *Celsr1* mutant phenotype. To identify partners of Celsr1 in cell fate determination, we used a gene candidate approach. As *Celsr1* mutants display some ASD features, we used the Simons Foundation Autism Research Initiative (SFARI) database together with the gene expression database genepaint (www.genepaint.org). Of 648 genes listed in SFARI, 63 were involved in both ASD and ADHD. 33 of them were expressed in the frontal cortex at e14.5, and 11 were detected in the VZ. Among those genes, *Ankrd11*, *Auts2*, *Mecp2*, and *Zbtb20* were regulated by RA in AP cultures *in vitro*. We selected *Auts2* for further investigations because this gene has been invariably associated with microcephaly in humans, whereas mutations in *Ankrd11*, *Mecp2* and *Zbtb20* can also lead to macrocephaly. Real time qRT-PCR experiments indicated that *Auts2* was downregulated in *Celsr1*-deficient mice and rescued by *in utero* supplementation of RA. Immunofluorescence analysis confirmed that, in addition to its documented expression in the cortical plate, Auts2 was expressed by neurogenic AP. Finally, *in utero* electroporation of *Auts2* in *Celsr1*$^{-/-}$ embryos at e14.5, when neurogenesis defect is detectable in the entire telencephalon, was sufficient to counteract the lack of *Celsr1* and to restore neurogenesis to normal levels. To investigate further the function of Auts2 in AP fate decision and cortical development, we overexpressed it in cultures of AP isolated from e14.5 cortex. Increasing Auts2 levels *in vitro* increased the differentiation of AP in proliferative conditions as indicated by the lower number of Sox2–positive cells in *Auts2–Gfp* transfected AP compared to *Gfp* transfected AP. It also accelerated the appearance of Tuj1-positive neurons in differentiation conditions. Given that *in vitro*, Auts2 increases differentiation of AP, we investigated its effect when overexpressed in the developing neocortex. To this end, we electroporated *Auts2* cDNA *in utero* at e14.5. This increased the delamination of AP from the VZ at e15.5, confirming the role of Auts2 in AP differentiation. The number of basal mitoses (in the SVZ) was increased suggesting that Auts2 promotes the differentiation and proliferation of BPs. In line with this, the number of Tbr2–positive cells deriving from electroporated AP increased, whereas that of Pax6–positive cells in and above the SVZ (i.e. basal radial glia) was not affected. As a consequence, the SVZ was enlarged. Considering that increased number of BP could be involved in expansion and gyrification of the brain, we overexpressed *Auts2* in e14.5 cortices and analyzed them at e17.5 and P1. The higher production of BP and the sustained enlargement of the SVZ improved the production of neurons and led to a marked thickening of upper layers and folding of the neocortex at P1.

Collectively, our results show that Celsr1 sculpts the basal processes of AP and mediate their communication with meninges. Celsr1–depleted AP have fewer basal processes and endfeet, reduced expression of *Aust2,* a transcriptional activator involved in neurological diseases such as ASD (autism spectrum disorder), and produce less BP and neurons. Finally, the forced expression of *Auts2* in the cerebral cortex of wild type embryos enlarges dramatically the SVZ, enhances the production of neurons, and induces the folding of the murine cortex. Those data are presently drafted and will be shortly submitted for publication

3. PCP genes in the gliogenic switch

Previous *in situ* hybridization studies showed that *Celsr3* is specifically expressed in postmitotic neurons and BP but not in AP, whereas *Fzd3* is widely expressed in both NPCs and neurons. We have examined PCP mutant brains at a late embryonic stage (e18.5), using layer–specific markers Tbr1 (layer 6), Ctip2 (layers 5–6) and Satb2 and Cux1 (layers 2–4). Compared with control samples, the number of Ctip2^{+} and Satb2^{+} cells was increased in *Celsr3* and *Fzd3* mutant cortices, with increased thickness of the cortical plate. This was confirmed in Tbr1 and Cux1–stained preparations. Of note, despite increased cortical neuron numbers, the border between Satb2 and Ctip2–positive layers was sharply defined in mutant cortex, indicating that radial migration and lamination were unaffected. The similar cortical defects in *Celsr3* and *Fzd3* mutants are therefore probably due to inactivation of Fzd3 in immature cortical neurons or BP, rather than in AP. To ascertain this, we produced conditional *Fzd3$^{f/-}$; Nex–Cre* mutant animals (*Fzd3$^{Nex\text{-}cKO}$*), in which *Fzd3* is specifically inactivated upon expression of *Nex–Cre* in cortical excitatory neurons. In *Fzd3$^{Nex–cKO}$* animals, increased production of DL and UL cortical neurons was observed, like in *Celsr3$^{-/-}$* and *Fzd3$^{-/-}$* mutant embryos. To estimate the number of AP in mutant and control samples, we used the AP marker Pax6 and found a significant decrease of the number of Pax6^{+} cells in *Celsr3$^{-/-}$*, *Fzd3$^{-/-}$*, and *Fzd3$^{Nex\text{-}cKO}$* mutant versus control samples. As cortical neurons derive from both AP and BP, we wondered whether the number of BP, which express Celsr3 at lower levels than neurons, was also affected. Using IHC with the BP marker Tbr2, we found that the number of BP was moderately decreased in *Celsr3$^{-/-}$* , *Fzd3$^{-/-}$* and *Fzd3$^{Nex\text{-}cKO}$* mutants versus control cortex. These observations of an excess of both DL and UL cortical neuron numbers, with depletion of NPC numbers suggest a defective feedback regulation from neurons.

We looked at earlier time–points to determine when the phenotype of increased neurogenesis was first observed, and found that the relative increase in mutants was first noticed at e15.5 and maintained at later stages. To define further the increased production of DL neurons in mutants, BrdU was injected at e13.5, and cells with strong nuclear BrdU labeling were counted. The proportions of BrdU^{+} cells expressing Tbr1 or Satb2 over the total number of BrdU^{+} cells was determined. The results showed that in *Celsr3* and *Fzd3* mutant cortex, the ratio of BrdU^{+} Tbr1^{+} to all BrdU^{+} cells was increased, whereas the proportion of BrdU^{+} cells that expressed the UL marker Satb2 was decreased in both *Celsr3* and *Fzd3* mutant samples. Several mechanisms could account for the observed increase of neuron number. It has been proposed that thalamocortical afferents influence neurogenesis in some context. To test a potential role of thalamic fibers, we examined *Celsr3$^{Dlx\text{-}cKO}$* mice in which these fibers do not develop and confirmed that the numbers of DL and UL neurons were similar to controls, arguing against a role of thalamic fibers in the phenotype. Another possibility could be differential cell death in mutant and control brains. To assess whether apoptosis was involved in the cortex of *Celsr3* and *Fzd3* mutants, we used immunohistochemistry (IHC) with activated Caspase–3 (aCas3), and found very few aCas3 positive cells, with no difference between control, *Celsr3* and *Fzd3* mutant samples at e14.5 and e16.5. A third possibility for increased neuron numbers could be premature NPC cycle exit and/or changes in cell cycle length. To estimate NPC cell cycle exit, we used BrdU and Ki67 double IHC to estimate cell cycle exit rates. We found no differences between control and mutant samples injected with BrdU at e12.5 and examined at e13.5. By contrast, in samples injected at e13.5 and studied at e14.5, and in those injected at e14.5 and processed at e15.5, the cell cycle exit rate was significantly increased in *Celsr3* and *Fzd3* mutants as compared to controls. To compare cell cycle length, we injected BrdU followed by EdU after 1.5 hour, and estimated S–phase and cell cycle length by BrdU and EdU double staining (30 min after EdU injection). No differences were found between control, *Celsr3* and *Fzd3* mutant cortex.

In the mouse necortex, at e16.5–E17.5 the generation of neurons declines and that of glial cells begins, the so-called “gliogenic switch”. We considered the possibility that the observed increase of cortical neurons may coincide with a delayed or decreased generation of glial cells. Cortical astrocytes and

oligodendrocytes are derived from Olig2^{+} glial precursor cells. We compared Olig2 expression in control and mutants and found that Olig2^{+} cells were more abundant in control than in *Celsr3* and *Fzd3* mutants at e17.5 and at birth. This indicates that neurons are generated at the expense of glial cells. To confirm this, we used GFAP IHC at postnatal days (P) 0 and P2. The intensity of GFAP staining was strongly decreased in mutant cortices, as was the concentration of GFAP, estimated using western blotting. To assess further the relative production of neurons and glia, we used triple IHC for BrdU, Satb2 to label late–generated neurons, and Olig2 to label glial precursors. We found that the ratio between glial cells and neurons decreased in *Celsr3*, *Fzd3* and *Fzd3*$^{Nex\text{-}cKO}$ mutant versus control samples. These data suggest that the neurogenesis may be prolonged or increased, and gliogenesis delayed or defective in mutant animals.

To explore mechanisms, we used western blotting with antibodies to phosphorylated Stat3, a readout of Jak-Stat signaling, phosphorylated Erk1 and 2, and phosphorylated Smads, an index of Bmp signaling. No difference was found between control, *Celsr3* or *Fzd3* mutants, suggesting that those signaling pathways are unaffected by inactivation of Celsr3 or Fzd3 in cortical neurons. We next focused on Notch signaling, a master regulator of neurogenesis and gliogenesis. We tested Notch activation at different embryonic stages, from e14.5 to e18.5, by IHC and western blotting with an antibody against the N-terminal epitope of the Notch1 intracellular domain (actN1) generated upon cleavage by the gamma secretase complex. IHC at e14.5 and e16.5 disclosed specific actN1 immunoreactivity in AP but no specific signal in postmitotic neurons. The signal was consistently and strongly decreased in *Celsr3* mutant compared to control cortex. Western blot analysis at E18.5 confirmed the downregulation of actN1 signal in *Celsr3* and *Fzd3* mutant versus control samples. Imprtantly, *Notch1* mRNA levels, estimated by qRT–PCR, remained unchanged, indicating that the modification of actN1 signals did not reflect decreased *Notch1* gene expression. In addition, transcripts of the Notch downstream effectors *Hes1*, implicated in neurogenesis, and *NFIA*, implicated in gliogenesis, were downregulated in both *Celsr3* and *Fzd3* mutant embryonic cortex, providing another independent evidence that Notch signaling is altered in PCP mutants.

What mechanisms could account for decreased Notch1 activation in *Celsr3* and *Fzd3* mutants? To assess the role of Notch ligands, we used an RNA–Sequencing to compare transcriptional profiles in control, *Celsr3* and *Fzd3* mutant cortical samples at e16.5. Among Notch ligands, *Dll1* mRNA was strongly expressed in all samples, whereas *Dll3*, *Dll4*, *Jag1* and *Jag2* were moderately represented. *Jag1* was the sole mRNA significantly downregulated in both *Fzd3* and *Celsr3* mutant samples. It was therefore a key candidate selected for further studies. *Jag1* mRNA expression was widespread, detected in VZ/SVZ as well as in postmitotic CP neurons, and was sharply downregulated at e14.5, e16.5 and E18.5 in *Celsr3*$^{-/-}$ mutant compared to control cortical samples. Using triple IHC with antibodies to Jag1, the neuronal marker Ctip2 and the BP marker Tbr2, Jag1 protein was clearly detected in immature neurons in the IZ and deep CP, with little colocalization with BP. Importantly, Jag1 immunoreactivity in the IZ and deep CP was specifically downregulated in *Celsr3*$^{-/-}$ mutant samples. Jag1 immunoreactivity in the IZ and deep CP was also reduced in *Fzd3*$^{-/-}$ mutant compared to control tissue.

If decreased Jag1 expression is implicated in diminished Notch activation and gliogenesis, its overexpression should rescue the phenotype. To test this, we electroporated a plasmid encoding Jag1 under the Cdk5r promoter that drives expression in neurons but not AP. Electroporation was carried out at e13.5, and samples were collected at e18.5 and stained with anti–Jag1 to verify overexpression, anti actN1 to check Notch activation, and anti–Olig2 and antiSatb2 to assess the gliogenesis switch. When Olig2 and Satb2 expression in the electroporated area were compared with that in the contralateral side, a significant increase in the density of Olig2 positive cells, together with a decreases in the density of Satb2 positive neurons and in the actN1 signal in VZ, were seen. We then asked whether Wnt proteins, which bind to Fzd receptors and are known to modify Jag1 expression, could be involved in Jag1

downregulation in *Celsr3* and *Fzd3* mutants. Our RNA–Seq results showed that Wnt7b and Wnt7a are by far the most abundant Wnt factors in the embryonic cortex, followed by Wnt5a, whereas other Wnt factors are expressed at very low, mostly undetectable levels. We tested the putative role of Wnt7 using primary cultures of cortical neurons from E14.5 control, *Fzd3* and *Celsr3* mutant embryos. After one day *in vitro*, neurons were placed in serum–free medium for 6 hours, exposed to Wnt factors for 5 hours, and expression of selected mRNA was assessed by qRT–PCR. Wnt7a is known to have mixed, non-canonical and canonical activities, depending on context. To differentiate between those two activities, we compared the effect of Wnt7a and Wnt3a, a canonical ligand. We measured expression of *Axin2*, a beta-catenin downstream target, and *Jag1*. As predicted, *Axin2* mRNA expression was upregulated upon addition of Wnt3a in both control and *Fzd3* mutant neurons, and remained unaffected upon addition of Wnt7a, indicating that the latter activated preferentially non canonical signaling in embryonic cortical neurons. Importantly, upon Wnt7a treatment, *Jag1* expression was increased in control but not in *Fzd3* or *Celsr3* mutant neurons, whereas addition of Wnt3a induced *Jag1* expression similarly in control and *Fzd3* mutant neurons. To confirm that this effect of Wnt7a occurred via non canonical Wnt signaling with JNK as a downstream effector, we used the JNK inhibitor SP600125. Addition of SP600125 to cultured neurons counteracted the effect of Wnt7a, and prevented *Jag1* upregulation.

Altogether, these results identify a novel and previously uncharacterized feedback mechanism that regulates the shift from neurogenic to gliogenic fate of NPC. Expression of Celsr3 and Fzd3 in immature cortical neurons and possibly BP cells allows AP to respond to a JNK-dependent, non–canonical Wnt signaling, presumably generated by Wnt7a and Wnt7b. Wnt7a and Wnt7b are both expressed in the embryonic cortex, with peaks of expression in VZ for Wnt7a, and in intermediate zone and cortical plate for Wnt7b. The Wnt7/Celsr3/Fzd3 signal fosters expression of Jag1 in immature neurons, which activates Notch in AP and orchestrates their sequential production of deep and upper layer cortical neurons, followed by glia. A manuscript describing the findings is currently under review in Nature Communications.

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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 unknown, 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).

Studies using gene-targeted deficient and transgenic mice have established the role of multiple **inflammatory chemokines and cytokines** produced by both infiltrating immune cells and resident CNS glial cells in EAE pathology. These cytokines and chemokines orchestrate a pathogenic cascade leading to demyelination and axonal damage. A crucial factor controlling inflammatory responses in MS is **NF-κB**. Although NF-κB activation in peripheral immune cells is absolutely essential for the induction of EAE pathology, little is still known about the involvement of NF-κB in the inflammatory reactions locally in the CNS (Mc Guire *et al*. (2013) Trends Mol. Med. 19:604). Although we could previously establish a brain-specific contribution of IKK-dependent NF-κB activation in the pathology of EAE (van Loo *et al.* (2006) Nat. Immunol. 7:954; van Loo *et al*. (2010) Mol. Endocrinol. 24:310; Raasch *et al*. (2011) Brain 134:1184), the specific role and contribution of NF-κB signaling in the different neuronal cell types (neurons, astrocytes, oligodendrocytes and microglia) for the pathology of MS is still unclear and subject of investigation in our research group.

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: 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 (Zhang and Kaufman (2008) Nature 454:455), 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) Nat.

Neurosci. 12: 379). Notably, IFN-γ exerts protective activities through the activation of the PERK-eIF2α pathway in oligodendrocytes (Lin *et al*. (2007) J. Clin. Invest. 117: 448). On the other hand, IFN-γ has suppressive activity on oligodendrocyte regeneration, inhibiting remyelination in MS and EAE demyelinated lesions (Lin *et al*. (2006) Brain 129: 1306). 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) Nat. Neurosci. 12: 379). 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) Nature 503: 272).

Together, with our studies we aim to better understand the contribution of NF-κB, autophagy and UPR signaling in the development and progression of CNS inflammation and demyelination. The basic approach is to genetically manipulate genes coding for proteins essential for NF-κB, 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 NF-κB, 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. It is 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 understand 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 project years 1-2

1.1. Methodology

Conditional 'floxed' mice targeting NF-κB signaling (IKK2^{FL}), autophagy (Atg16L1^{FL}, OPTN^{FL}) and individual UPR signaling pathways (XBP1^{FL}, IRE1α^{FL}, and PERK^{FL}) have been obtained from different sources. Also the different Cre transgenic lines needed for CNS targeting are available for our research: these include 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 NF-κB, ER stress and autophagy in the immunopathology of MS, we make use of the experimental MS model EAE, which can be induced in C57BL/6 mice by immunization with myelin oligodendrocyte glycoprotein (MOG). Next to EAE, brain-specific demyelination is also 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, *in vivo* studies are complemented by *in vitro* studies on primary cells derived from the different tissue-specific knockout mice that have been established, allowing biochemical studies.

1.2. UPR signalling and autophagy 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 clearly induces activation of autophagy genes (Figure 1) and ER stress response genes (Figure 2) in the corpus callosum.

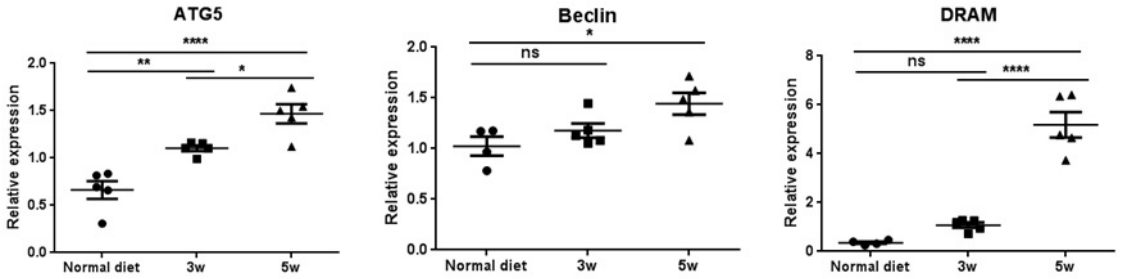

Figure 1. Cuprizone-induced expression of autophagy genes. Expression of autophagy markers was assessed through qPCR on corpus callosum lysates isolated from wild-type C57BL/6 mice either or not treated with cuprizone for 3 and 5 weeks. Results are presented as mean values ± s.d.

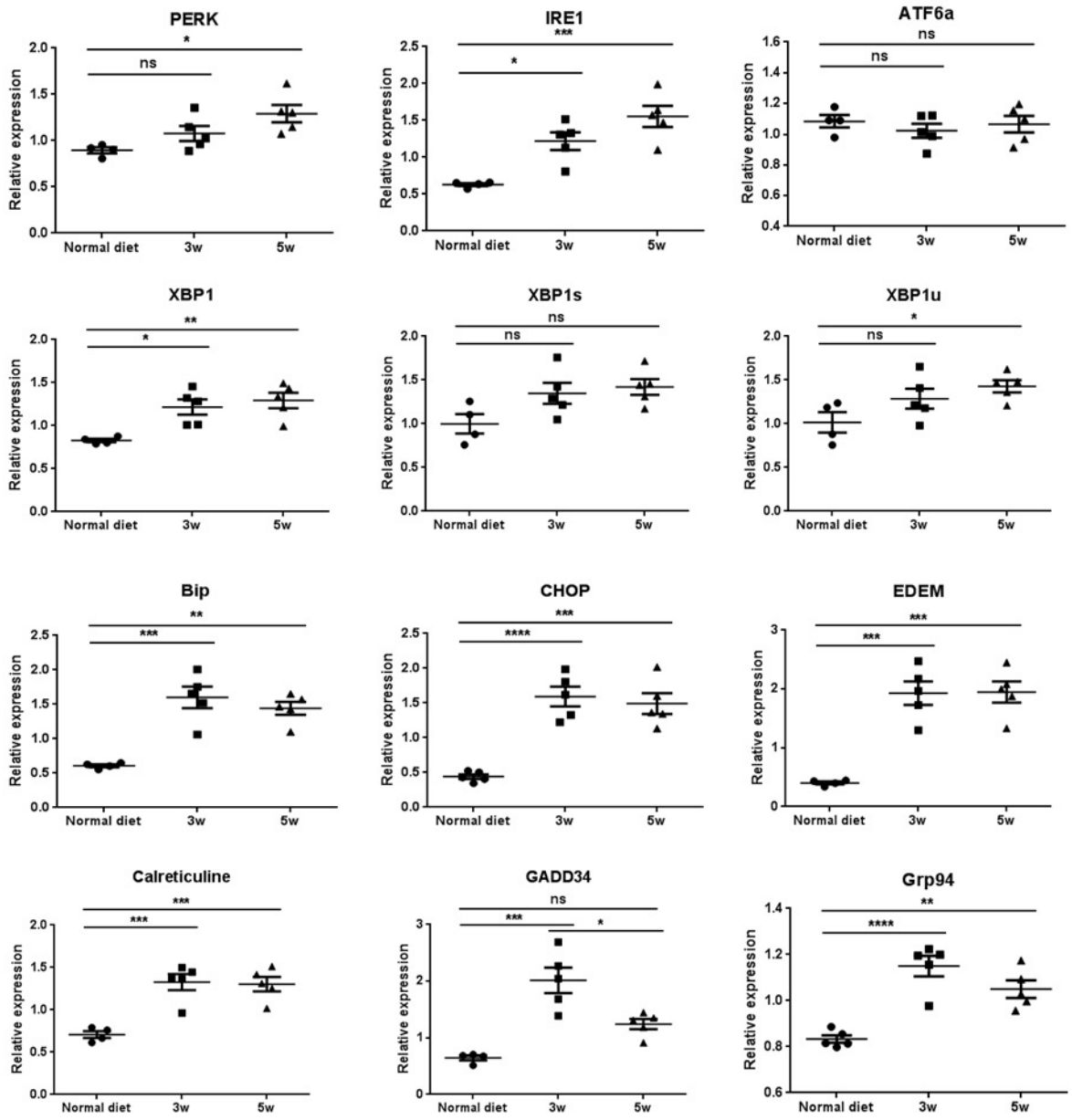

Figure 2. Cuprizone-induced expression of UPR genes. Expression of ER stress markers was assessed through qPCR on corpus callosum lysates isolated from wild-type C57BL/6 mice either or not treated with cuprizone for 3 and 5 weeks. Results are presented as mean values ± s.d.

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 3). Loss of XBP-1 transcriptional activity may result in constitutive IRE-1α signalling and IRE-1α dependent degradation (RIDD) of specific mRNAs, as previously shown (Hur *et al.* (2012) J. Exp. Med. 209: 307; Osorio *et* al. (2014) Nat. Immunol. 15: 248), explaining observed phenotype. 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) Genes Dev. 23: 2294), 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. These hypotheses are currently under investigation. For this, XBP1^{CNS-KO} mice are being crossed to IRE-1α^{CNS-KO} mice and Atg16L1^{CNS-KO} mice, and the response of these CNS-specific double knockout mice to cuprizone will be investigated.

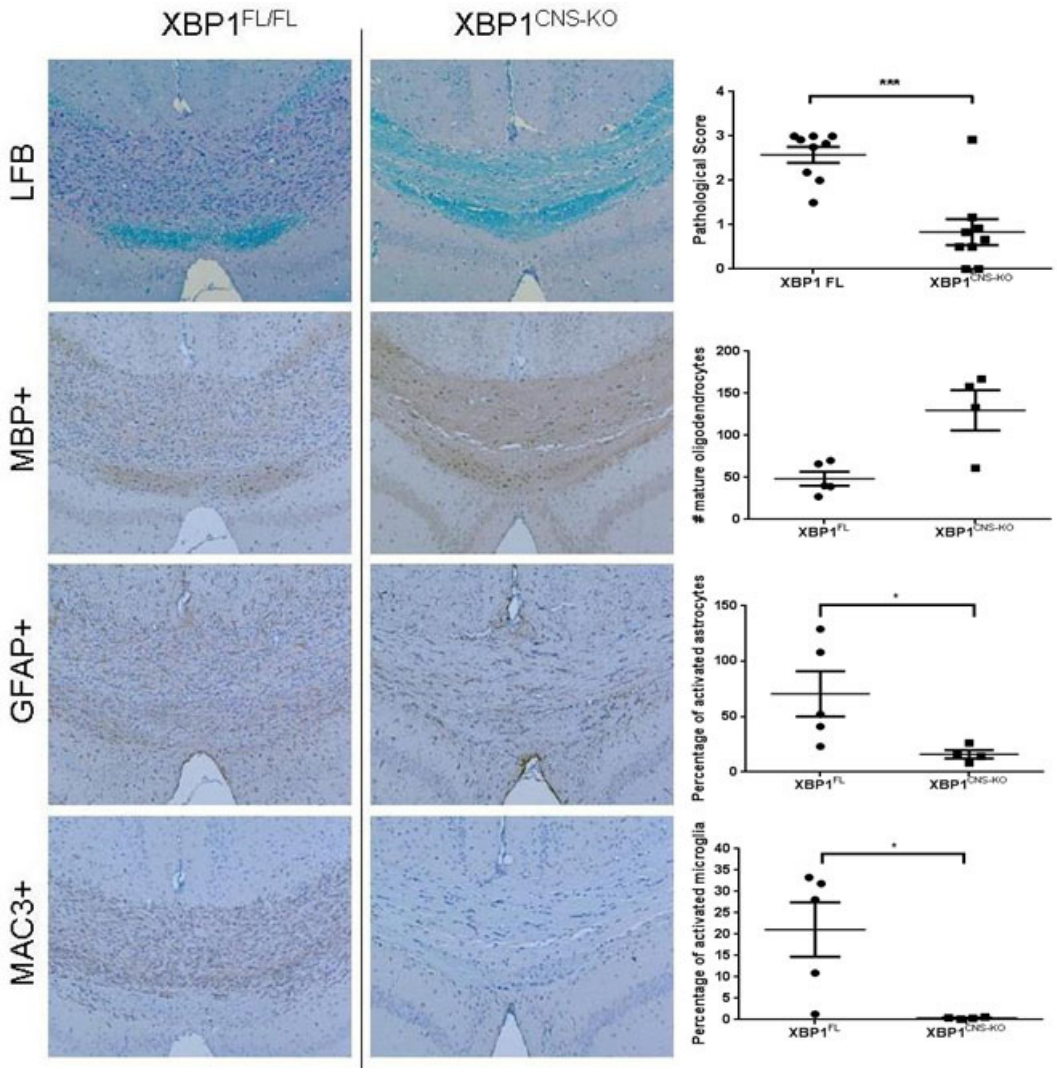

Figure 3. Cuprizone-induced demyelination is reduced in XBP1^{CNS-KO} mice. Representative pictures of coronal sections of the corpus callosum (left) and quantification (right) for degree of demyelination (LFB), oligodendrocyte numbers (MBP), astrogliosis (GFAP) and microgliosis (Mac3) in XBP1^{CNS-KO} mice and XBP1^{FL} littermates after 5 weeks of cuprizone treatment.

1.3. XBP1- and IRE1α-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 IRE1α– and XBP1-dependent UPR signalling in CNS inflammation, we generated CNS-specific IRE1α and XBP1 deficient mice and subjected them to EAE. XBP1^{CNS-KO} mice, however, developed EAE pathology to the same extent as control littermates (Figure 4). Also, on spinal cord histology nor on spinal cord gene expression, no significant differences could be observed (data not shown). In case of IRE1α^{CNS-KO} mice, however, a lot of variation between different mice within one group was observed, and many mice did not develop a significant degree of disease probably due to the genetic background of the IRE1α line. Therefore, we now backcrossed these mice into a C57BL/6 background and mice are currently challenged in EAE. Since XBP-1 deficiency may upregulate IRE-1α signalling and IRE-1α dependent degradation (RIDD), as described above, also XBP1-IRE1α double deficient mice have been generated and will be subjected to EAE.

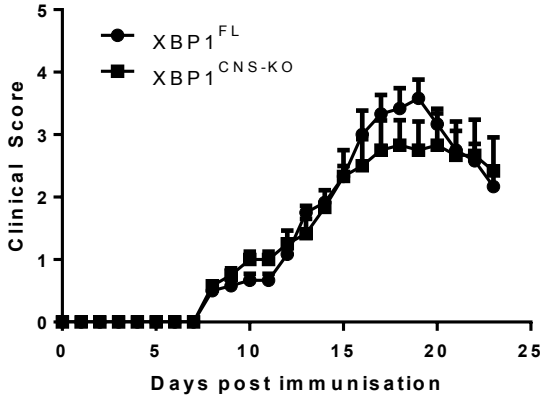

Figure 4. Clinical disease score of XBP^{FL}(n=6) and XBP^{CNS-KO} (n=6) littermate mice after immunization with MOG peptide. Results are presented as mean values ± s.e.m.

1.4. Atg16L1-dependent autophagy in CNS and EAE

One essential signaling mediator in autophagy signalling is the protein Atg16L1. Although the primary role of autophagy is the adaptation to starvation, autophagy is also thought to be essential for the normal turnover of cytoplasmic proteins. As such, autophagy is thought to be a protective mechanism. Moreover, in inflammatory conditions, autophagy is often induced, probably as a compensatory mechanism.

Since Atg16L1 knockout mice are not viable, we generated a floxed *Atg16L1* mouse allowing cell type-specific Atg16L1 deletion. For the study of autophagy in brain and EAE pathology, Atg16L1 floxed mice were first crossed with the NestinCre line in order to generate CNS-specific Atg16L1 knockout mice. These mice are viable, however, progressively develop motor and behavioral deficits. The gross anatomy of the brain of these mice was normal. Histological examination, however, revealed degenerative changes in specific neuronal populations in Atg16L1^{CNS-KO} mice, most evident in the cerebellum demonstrating severe loss of Purkinje cells (Figure 5). Further studies will be done in order to characterize the cause of neurodegeneration in these mice. Because of the spontaneous neurodegenerative phenotype of Atg16L1^{CNS-KO} mice, these mice will not be challenged in EAE. However, Atg16L1-specific signalling and autophagy in microglial cells will be studied in the context of neuroinflammation and EAE. Indeed, microglia play crucial roles in neuroinflammation, and autophagy in glial cells may play important roles in responding to stress in MS. For this, Atg16L1$^{microglia-KO}$ mice have been generated and will be subjected to EAE.

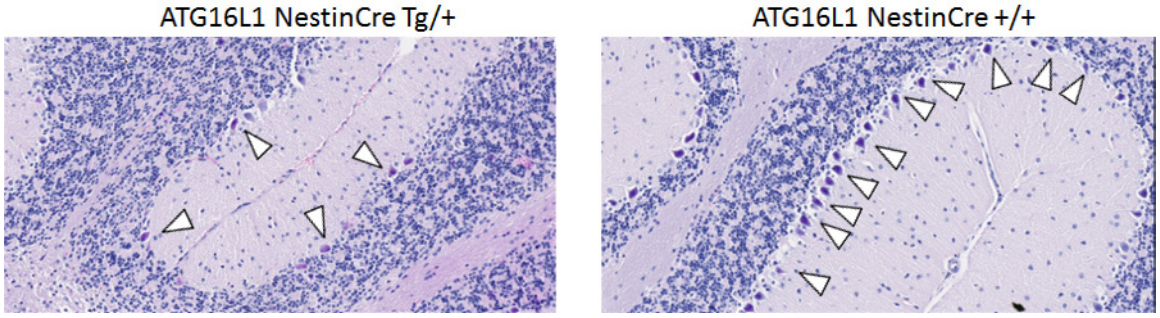

Figure 5. Neuronal degeneration in Atg16L1^{CNS-KO} mice. H&E stained sections of cerebellum from control (Atg16L1 NestinCre +/+) and Atg16L1^{CNS-KO} (Atg16L1 NestinCre Tg/+) mice at three months of age. Purkinje cells are indicated with arrowheads.

1.5. Optineurin in EAE

Optineurin (OPTN) is ubiquitously expressed ubiquitin-binding protein that has been implicated, through genetic studies in patients, in amyotrophic lateral sclerosis (ALS) and other neurodegenerative diseases. We recently generated OPTN deficient mice and identified the *in vivo* importance of OPTN in inflammatory signalling and autophagy. In agreement with previous *in vitro* data, we could show a role for OPTN in the activation of TBK1 and the production of type I IFNs, establishing OPTN as a positive regulator of IRF3 signalling and IFN- production (Slowicka *et al.* (2016) Eur. J. Immunol. in press). Interestingly, TBK1 controls T cell activation and migration during neuroinflammation (Yu *et al.* (2015) Nat. Commun. 6: 6074), suggesting a role for OPTN in this process.

To determine whether OPTN plays a role in EAE pathogenesis, we induced EAE in control heterozygous (OPTN$^{-/+}$) and OPTN deficient (OPTN$^{-/-}$) littermate mice. Upon EAE induction, OPTN$^{-/-}$ mice were slightly protected from disease development and recovery, not from disease initiation, when compared to OPTN$^{-/+}$ mice (Figure 6). These preliminary observations suggest that OPTN, being an autophagy receptor and regulator of inflammatory signalling, could contribute to EAE progression. We are currently challenging a second group of mice in EAE to establish the importance of OPTN in neuroinflammation and EAE pathology.

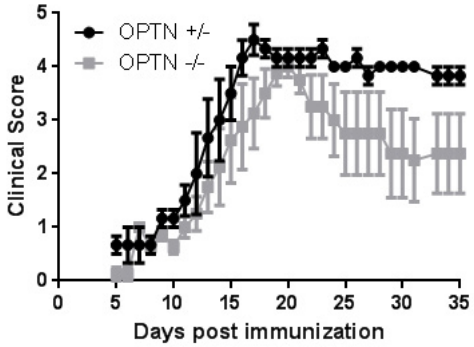

Figure 6. Clinical disease scores of OPTN$^{-/-}$ (n=4) and OPTN$^{+/-}$ (n=3) mice after immunization with MOG peptide. Results are from three independent experiments and presented as mean values ± s.e.m.

1.6. Future studies

Besides follow-up studies as described above, new mouse lines with specific defects in PERK-dependent UPR signalling in specific 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.

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Progress report of the research group of

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

1. 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 (Brennand et al., 2015; Suzuki and Vanderhaeghen, 2015). 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 this year are detailed below:
1. Exploring new ways to repair the diseased cortex.
2. Modelling human neurodevelopment diseases.

2. Progress Report.

2.1. 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:

- 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, **Vanderhaeghen P**.
  Neuron (2015), 85(5):982-97.

2.2. 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 (Hasche et al., unpublished data).

In parallel, we have used our protocols of corticogenesis, in collaboration with the groups of Prof. Van Hesch (KU Leuven) and Muotri (UCSD) to explore the mechanisms of pathogenesis of another class of neurodevelopmental diseases caused by the duplication of MecP2, revealing altered patternns of neuronal differentiation and synaptogenesis (Nageshappa et al., 2015). Finally in collaboration with the group of Prof Verfaillie (KU Leuven), the application of our protocols of corticogenesis to a neurodegenerative condition (fronto-temporal dementia) caused by progranulin mutation, revealed early developmental phenotypes in affected neurons, thus leading to novel insights on ths disease (Raitano et al., 2015).

This set of results is part of the following publications:

– Altered neuronal network and rescue in a human MECP2 duplication model.
  Nageshappa S, Carromeu C, Trujillo CA, Mesci P, Espuny-Camacho I, Pasciuto E, **Vanderhaeghen P**, Verfaillie CM, Raitano S, Kumar A, Carvalho CM, Bagni C, Ramocki MB, Araujo BH, Torres LB, Lupski JR, Van Esch H, Muotri AR.
  Mol Psychiatry (2015), doi: 10.1038/mp.2015.128.
– Restoration of Progranulin Expression Rescues Cortical Neuron Generation in an Induced Pluripotent Stem Cell Model of Frontotemporal Dementia.
  Raitano S, Ordovàs L, De Muynck L, Guo W, Espuny-Camacho I, Geraerts M, Khurana S, Vanuytsel K, Tóth BI, Voets T, Vandenberghe R, Cathomen T, Van Den Bosch L, **Vanderhaeghen P**, Van Damme P, Verfaillie CM.
  Stem Cell Reports (2015) 13;4(1):16-24.
– Microcephaly-associated mutations in *ASPM* gene lead to defective human corticogenesis through overactivation of Wnt signaling.
  Anja Hasche, Matteo Piumatti, Daisuke H. Tanaka, Adèle Herpoel, Angéline Bilheu**,** Harmen van Benthem, Isabelle Pirson, Gert Matthijs, Kathelijn Keymolen, Julie Désir, Pierre Gressens, Sandrine Passemard, Marc Abramowicz, and **Pierre Vanderhaeghen**.
  Neuron, in revision.

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4. FMRE-GSKE 2014-2016 / Vanderhaeghen Pierre, I.R.B.H.M. U.L.B. / Most significative publications published in 2015

- 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 , **Vanderhaeghen P**.
  Neuron (2015), 85(5):982-97.
  IF: 15.9
- Altered neuronal network and rescue in a human MECP2 duplication model.
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  Mol Psychiatry (2015), doi: 10.1038/mp.2015.128.
  IF: 14.4
- Restoration of Progranulin Expression Rescues Cortical Neuron Generation in an Induced Pluripotent Stem Cell Model of Frontotemporal Dementia.
  Raitano S, Ordovàs L, De Muynck L, Guo W, Espuny-Camacho I, Geraerts M, Khurana S, Vanuytsel K, Tóth BI, Voets T, Vandenberghe R, Cathomen T, Van Den Bosch L, **Vanderhaeghen P**, Van Damme P, Verfaillie CM.
  Stem Cell Reports (2015) 13;4(1):16-24.
  IF: 5.6
- Is this a brain which I see before me? Modelling human neural development with pluripotent stem cells.
  Suzuki I, and **Vanderhaeghen P**.
  *Development (*2015),142(18):3138-50. doi: 10.1242/dev.120568.
  IF: 7.8
- Creating Patient-specific Neural Cells for the In Vitro Study of Brain Disorders.
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