

Quantification of intraneuronal transport by fluorescent nanodiamond tracking. Application to the screening of the functional impact of neuropsychiatric disease-related genetic risk factors.

Simon Haziza

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THESE DE DOCTORAT de L'UNIVERSITE PARIS-SACLAY

Préparée à L'ECOLE NORMALE SUPERIEURE DE CACHAN

Ecole Doctorale N°572 Ondes et Matieres

Spécialité de doctorat: Optique et Photonique

Par

M. Simon HAZIZA

Quantification du transport intraneuronal par suivi de nanodiamants fluorescents. Application à l'étude de l'impact fonctionnel de facteurs de risque génétiques associés aux maladies neuropsychiatriques.

Thèse présentée et soutenue à l'ENS de Cachan, le 26 Novembre 2015:

Composition du Jury :

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"El gran libro, siempre abierto y que tenemos que hacer un esfuerzo para leer, es el de la Naturaleza, y los otros libros se toman a partir de él, y en ellos se encuentran los errores y malas interpretaciones de los Hombres."

Antoni GAUDÍ

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GENERAL INTRODUCTION

Neuropsychiatric disorders, such as Autism Spectrum Condition (ASC) or schizophrenia (SZ), and neurodegenerative diseases, such as Alzheimer's disease (AD), represent an enormous burden to afflicted individuals and their families. Contrary to rare brain diseases which impact less than 0.001% of people, these common brain diseases are striking more than 1% of the world-wide population and their heritability is also very high with more than 50% chance of transmission (Table 0.1). Some of these diseases begin early in life, are life-long, and damage the patients' self-perception, productivity and relationship to others. Mental disorders also have a cost for the society which have been estimated to about 800 billion euros per year in Europe in 2010 (Olesen et al., 2012) and the average cost per European citizen was evaluated around \in 5.550 per year. Importantly, many of brain diseases have no morphological phenotypes, such as Alzheimer's disease in which predominant morphological abnormalities (such as plaques or tau entanglement) can be used as pathological diagnosis.

Name	Life prevalence	Heritability	Essential characteristics	Notable feature
Alzheimer's disease	0.132	0.58	Dementia, defining neuropathology	Of the top ten causes of death in the United States, Alzheimer's disease alone has increasing mortality
Attention-deficit hyperactivity disorder (ADHD)	0.053	0.75	Persistent inattention, hyperactivity, impulsivity	Costs estimated at ~\$US100×10 ⁹ per year
Alcohol dependence (ALC)	0.178	0.57	Persistent ethanol use despite tolerance, withdrawal, dysfunction	Most expensive psychiatric disorder (total costs exceed US\$225×10º per year)
Anorexia nervosa	0.006	0.56	Dangerously low weight from self-starvation	Notably high standardized mortality ratio
Autism spectrum disorder (ASD)	0.001	0.80	Markedly abnormal social interaction and communication beginning before age 3	Huge range of function, from people requiring complete daily care to exceptional occupational achievement
Bipolar disorder (BIP)	0.007	0.75	Manic-depressive illness, episodes of mania, usually with major depressive disorder	As a group, nearly as disabling as schizophrenia
Major depressive disorder (MDD)	0.130	0.37	Unipolar depression, marked and persistent dysphoria with physical and cognitive symptoms	Ranks number one in the burden of disease in the world
Nicotine dependence (NIC)	0.240	0.67	Persistent nicotine use with physical dependence (usually cigarettes)	Major preventable risk factor for many diseases
Schizophrenia (SCZ)	0.004	0.81	Long-standing delusions and hallucinations	Life expectancy decreased by 12–15 years

Table 0.1: Key features of 9 mental disorders. Life prevalence, heritability and specific features are shown. From (Sullivan et al., 2012).

However, molecular biomarkers of each brain diseases are lacking and objective medical diagnosis and efficient drug treatments are not available yet. Fortunately, common brain diseases, such as SC or ASC, involve the interaction of a large number of genes (polygenicity) in combination with non-genetic risk factors, such as environmental factors like child mistreatment (McCarroll en Hyman, 2013). Therefore, genetic information can provide invaluable insight on brain disorders for four reasons that are intrinsically linked: to understand and diagnose each brain disease as well as to design and follow response to drug treatments. To diagnose neuropsychiatric disorder, psychiatrists use standard classification, namely *the Diagnostic and Statistical Manual of Mental Disorders*, 5th Edition (DSM-5) (2013). This manual is based on well-stratified and characterized psychiatric disorders. However, recent data from genetics, cognitive neuroscience or imaging argue that psychiatric disorders in terms of arbitrary lists of symptoms, and diagnosis is thus biased (McCarroll et al., 2014). Contrary to other common polygenic diseases such as cancer or immunologic diseases, neurobiologists studying brain diseases cannot, ethically and practically, sample the human brain for bench experiments. Thereby, genetic analysis will play a key role to improve and refine clinical diagnosis of individuals with mental illnesses.

While understanding the complexity of mental disorders is an important goal of neurogenomics, providing better treatments is also crucial. Current psychiatric medications are based on drugs discovered between 1949 (lithium) and 1951-1957 (neuroleptics: 1951, chlorpromazine, Henri Laborit & Pierre Deniker, Sainte-Anne Hospital, Paris; 1957, haloperidol, Paul Janssen) which can only treat a subset of symptoms such as antipsychotic drugs antagonizing D2 dopamine receptors (McCarroll et al., 2014). And no significant improvement has been done since then. What slow down the discovery of new therapeutic agents is the lack of insight into the molecular mechanisms and anatomical basis of brain diseases. Across all levels of analysis in medicine, molecular data have proven to be the most important information to identify and validate drug targets and to discover biomarkers specific to each mental disorders (Hyman, 2012; Jack en Holtzman, 2013).

Altogether, the facts highlighted here stress the utmost importance to dissect the underlying neurobiology for each mental disorder to have a chance to objectively diagnose them and cure all afflicted individuals. The complex polygenic nature of brain diseases suggests that truly multidisciplinary approaches involving neurobiology, neurogenomics, chemistry and physics must be performed to identify relevant biological markers of each brain diseases. As a first step of this rising challenge, neurogenomics analysis can be used to filter out relevant biological pathway and eventually develop dedicated molecular diagnosis assay (Figure 0.1).



Figure 0.1: From Bed to Bench. A novel molecular diagnosis assay of neuropsychiatric disorders (right; from Munch, "Le Cri") based on intraneuronal tracking (middle) of Nitrogen-Vacancy-color center fluorescent nanodiamonds (right).

OUTLINE of the PhD MANUSCRIPT

This manuscript contains the research work carried out during my three years of PhD (October 2012 – September 2015). This work was supported by a public grant overseen by the French National Research Agency (ANR) through the "IDI 2015" project (Initiative Doctorale Interdisciplinaire) funded by the IDEX Paris-Saclay, ANR-11-IDEX- 0003-02. Most of the work was done at ENS Cachan in the Biophotonics team (PI Prof. François TREUSSART) before it moved to "Laboratoire Aimé Cotton" (LAC, CNRS UMR9188, Université Paris Sud and ENS Cachan) in Orsay, and in the Neuropathophysiology team (PI Prof. Michel SIMONNEAU) of "Centre de Psychiatrie et Neurosciences", Sainte-Anne Hospital, (CPN, INSERM UMR894, Université Paris Descartes). The LAC's team has developed the applications of nitrogen-vacancy (NV) color centers in diamond nanocrystals to fluorescence bioimaging. Other activities of LAC researchers on NV center comprise nanomagnetometry and quantum information processing. The main research topics of the CPN's team is the functional analysis of genetic mutations and the discovery of dysregulated gene networks based on functional genomics in the context of neuropsychiatric and neurodegenerative diseases.

This manuscript contains four chapters.

The **first chapter** details the complex polygenic architecture of mental disorders that combines common and rare genetic variants which synergistically impair specific biological pathways such as synaptic

plasticity. Then, two widespread approaches, namely electrophysiology and super-resolution nanoscopy, commonly used to address the question of the functional impact of genetic mutations associated to synaptopathies are described. Finally, we demonstrate the disease relevance of monitoring the intraneuronal transport, by using state-of-the-art bioinformatics meta-analysis of schizophrenia and ASC gene datasets.

The **second chapter** shows that fluorescent nanodiamond (fND) appears to be the probe of choice to record intraneuronal transport dynamics. This chapter starts with a brief review of organic and inorganic fluorescent probes potentially useful for such a study. Then, the production, physical properties and bio-applications of fNDs are described. Finally, we detail our experimental procedure to internalize fNDs inside neurons dissociated from mouse embryo, and to image motions of fND-containing cargos. Internalization pathway and cytotoxicity are also discussed.

The **third chapter** is dedicated to the quantitative analysis of fNDs trajectories and to the validation of the single particle tracking (SPT) assay. We first discuss the choice of the SPT software and the measurement of the localisation precision of our live-cell imaging setup. Then, we review common SPT analysis tools to study dynamical processes and we explain how we automatically detect the STOP and GO binary motion, characteristic of microtubule-based transport. Finally, we validate the intra and interexperiment stability of the "fND tracking assay" and we demonstrate the sensitivity of this approach to nanomolar concentrations of pharmacological compounds.

The **fourth chapter** is devoted to the application of the fND tracking assay to measure the functional impact on the intraneuronal transport of brain disease-related genetic risk factors. We first review the neurobiologists' Toolbox commonly used for *in vitro* and *in vivo* expression of brain disease-related genes. Then, we evaluate the techniques sensitivity to subtle changes impacting either the microtubule "tracks" (1st order perturbation), or the mitochondria (2^{sc} order perturbation), or the transcription machinery located in the nucleus (3rd order perturbation). Whether it is functionally close to the intraneuronal transport mechanism or far from it, we observe significant impairment of the "intraneuronal transport" when mimicking the effect of brain disease-related genetic risk factors. Altogether, we prove this nanoparticle-based technology to be sufficiently sensitive to screen the functional impact of genetic variants, paving the way for future development in translational nanomedicine.

CHAPITER 1

Genetic architecture of mental disorders and functional impact of genetic variants

Summary

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1.1 Introduction

This chapter draws the global framework of my PhD. It emphasizes the need to scrutinize the intraneuronal transport endophenotype in order to evaluate the functional impact of genetic mutations at the neuron scale, leading to the development of a novel molecular diagnosis assay. We first describe the **polygenic architecture** of brain diseases which relies on a complex combination of common variants of low penetrance

(i.e. effect) and rare variants of high penetrance, called "genetic risk factors" (McCarroll et al., 2014; Veltman en Brunner, 2012). Current genetic evidences converge on specific molecular complexes and pathways such as postsynaptic components of excitatory synapses (Fromer et al., 2014; McCarroll en Hyman, 2013; McCarthy et al., 2014; De Rubeis et al., 2014). The next part therefore details recent achievements in understanding the **functional impact of these genetic variants** in synaptic plasticity using electrophysiology and super-resolution imaging, with the description of some results I've obtained apart from my PhD project. Eventually, using state-of-the-art bioinformatics analysis on Schizophrenia and ASC gene datasets (Chang et al., 2015; Gilman et al., 2012), we demonstrate in the last part of this Chapter 1 that **abnormal endophenotypes** associated to these mental disorders might also be due to impairment of the **intraneuronal transport**.

1.2 Polygenic architecture of psychiatric diseases combining

common and rare genetic variants.

The failure of linkage studies (1980's and 1990's) and candidate-gene association studies (1990's and 2000's) in their effort to identify disease-associated genes in neuropsychiatric disorders started to highlight the polygenic architecture of mental illnesses and urged neurogeneticists to develop new approaches to tackle this challenge. Briefly, the first genetic approach aims at identifying genomic segment (*loci*) that are enriched within neuropsychiatric disorders in families with multiple affected individuals. Even though it did not work for ASC and Schizophrenia, this approach was successful for familial Alzheimer's disease. The later disease displays a Mendelian pattern of inheritance, with the finding of highly penetrant *loci*, for which *in vitro* mimicking leads to recent breakthrough in the field (Choi et al., 2014; Rovelet-Lecrux et al., 2006). The second genetic approach is based on the underlying biological knowledge of the disease and is thus hypothesis-driven and biased: once appropriate genes are selected, association studies are conducted to check if a particular allele of one gene is enriched in cohort of affected subjects compared to healthy control individuals. The traditional family-based genetic studies being unsuccessful for complex polygenic diseases, neurogeneticists shifted toward population-based genetic studies.

Since 2009, and thanks to innovation in sequencing technologies, unbiased analysis of the entire genome conducted on large cohorts of affected versus unaffected individuals has been demonstrated to be a powerful approach for the study of multifactorial brain diseases. Common-Variant Association Studies (CVAS, also referred to as GWAS for Genome-Wide Association Studies) and Rare-Variant Association Studies (RVAS) for the discovery of common variants of low penetrance and rare variants of high penetrance respectively, allow to decipher the underlying polygenicity of mental disorders (Figure 1.1 and Figure 1.2).

In this section, we address the polygenic architecture of mental disorders with the description of the concepts and the methods standardly used to unravel brain disease-related common and rare variants.



Figure 1.1: Polygenic architecture of mental disorders. Allele frequency represents a continuum in human populations, with cut-offs between 'common' and 'rare' being somewhat arbitrary. The frequency of common variants and the large range of frequencies of rare variants are displayed along with the methodologies used to detect different types of variations. From (McCarroll et al., 2014).

1.2.1 Genome-Wide Association Studies for common variants

GWAS have evolved over the last ten years into a powerful tool for investigating the genetic architecture of human diseases. Before going further, one needs to take a brief detour through human population history to understand the concept of Common Disease/Common Variants, and how CVAS studies are conducted (Bush en Moore, 2012; McCarroll en Hyman, 2013; McCarroll et al., 2014).

If more than 7 billion human beings inhabit the Earth today, only a far smaller number of people (few thousands individuals) of our species were living 5000 generations ago (i.e. 100 000 years ago). At each generation, several dozen of *de novo* mutation appeared (see section 1.1.2) and all of our 23 chromosomes pairs recombined each other. Therefore, after the filtration of natural selection, the dramatic expansion of human populations from smaller groups of ancestors has profoundly fashioned the patterns of variation that exist in human genomes in our modern society: the sequence variation (called "polymorphism") that was present in those small, ancestral populations is today common and found throughout the world. GWAS aims at evaluating such polymorphisms on thousands of genetic backgrounds and in diverse environmental contexts.

Human populations contain about 10 million common sequence polymorphisms and whole-genome sequencing has permitted these polymorphisms to be systematically catalogued, as by the 1000 Genomes Project (Durbin et al., 2010; Project et al., 2012). Moreover, due to the chromosomal recombination effects, some polymorphisms (the so called Single Nucleotide Polymorphism, SNPs) near one another on a chromosome are often transmitted together to the next generation without any chromosomal recombination between them. This phenomenon is called "linkage disequilibrium" and such a region is called "haplotype". These genomic segments, or haplotype, are typically tens of kilobases long and are called "locus" ("loci" if several) and encompass several genes (only one third span one gene) (Figure 1.2). Therefore, from this

knowledge, it is possible to genotype only a few hundred thousand well-selected SNPs across the genome and then statistically infer the others. Practically, to conduct such a GWAS study, around one million of DNA sequences (DNAseq_i for SNP_i + Σ (SNP_{infered})) are studied using a DNA microarray chip for each individual from the case/control cohorts (McCarroll et al., 2014).

Considering the concept of Common Disease/Common Variants, GWAS aims to identify alleles that are more common in individuals affected by common human disease than in unaffected ones. The first successful GWAS was published in 2005 (Klein et al., 2005), in which the authors investigated patients with age-related macular degeneration and only two SNPs were found significantly enriched in cases versus controls. Indeed, due to multiple hypothesis testing, GWAS demand a high level of statistical evidence for relationship to disease, with a typical threshold of $p = 5 \times 10^{-8}$ (Lander en Kruglyak, 1995). Consequently, larger cohorts are required to reach this level and to have a chance to detect a genetic variant. For this reason, GWAS applied to psychiatric disorders appeared for many years to be unsuccessful. For example, the most deeply studied neuropsychiatric disorders, namely Schizophrenia (SZ), did not allow establishment of sufficiently high number of patients and GWAS studies were always underpowered. As international collaborations around SZ, namely Schizophrenia Working Group of the Psychiatric Genomics Consortium (PGC), have expanded sample size, GWAS studies are finding far more genetic risk factors. Years after years, by increasing the size of cohorts, PGC uncovered 5 more *loci* in 2011 using 10,000 cases (Lee et al., 2012), then 22 more *loci* in 2013 (Ripke et al., 2013) and the up-to-date in 2014 showed a total of 108 *loci* using 36,989 cases and 113,075 controls (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

Such studies have a direct impact on the understanding of mental disorders because they are pointing out several individual genes for further biological and functional analysis. Indeed, GWAS are creating an initial molecular "short list" for Schizophrenia and in close future for Bipolar Disorder and ASD, each of them being currently underpowered (Anney et al., 2010; Weiss et al., 2009). Nevertheless, GWAS by itself failed to explain common mental disorders because the penetrance of common genetic variants is low, contrary to other components of the polygenic architecture of neuropsychiatric and neurodegenerative diseases (Figure 1.1 and Figure 1.2).

1.2.2 Rare and de novo variants: SNVs, Indels & CNVs

Apart from common genetic polymorphisms, which are inherited from ancestors (could have been rare at this time i.e. 100 000 years ago), recent genetic variants in the modern populations have arisen. These variants are rare and relatively unfiltered by natural selection, thus likely to have more deleterious effects. Rare variants can either be transmitted from parents with no natural selection or be *de novo* (Figure 1.1 and Figure 1.2) (Krumm et al., 2015; McCarroll et al., 2014). Predicting the pathogenicity of rare mutations in novel genes is particularly challenging and the identification of these mutations requires large (international) collaborations. However, it is greatly facilitated by the identification of recurrent mutations in patients with similar disease phenotypes, allowing detailed genotype-to-phenotype studies to be carried out.

The first clue of these rare variants came from **Copy Number Variants** (CNV) found in the genome of a non-negligible proportion of patients displaying either ASC or Schizophrenia. CNVs differ from Insertion-Deletion (indels) and Single-Nucleotide Variants (SNV also previously referred as SNP) based on their DNA size. When SNV correspond to only one nucleotide change, indels are small insertions or deletions of 1-1000 nucleotides whereas CNV are large insertions or deletions of more than 1000 nucleotides (Figure 1.2). As studies of CNVs have expanded, more than a dozen *loci* have been found in Schizophrenia at which large (>200 kb), recurring deletions or duplications are present in 0.1–1% of affected individuals but are 3–30 times rarer in the general population (Rees et al., 2014a). As described in (Sanders et al., 2011), large (500 kb) *de novo* CNVs found in the genome are observed several times more frequently in patients with autism than in controls. However, these CNVs are randomly distributed across all the genome and, only a small minority of the disease-associated CNVs are recurrent at specific sites within the genome (they are called recurrent CNVs; see section 1.4.2.). Moreover, a large number of genes are included in one CNV and therefore, it is difficult to determine if the damaging effects are mediated by a single gene or by interactions among genes within the CNV.



Figure 1.2. Common brain diseases are induced by many genetic risk factors that change the gene expression or function. These genetic variants can be classified into 4 types with respect to the number of nucleotide involved: SNV, InDel, CNV and GWAS Loci. Another classification can also rely on their occurrence frequency for a given disease: common, rare and de novo variants from highly frequent (more than 10 over 100) to lowly frequent (less than 1 over 1 million). GWAS Loci are the common variants whereas the other ones can be both rare and de novo variants. The penetrance of a genetic variant is inversely proportional to its frequency in the overall population.

As rare variants are not frequent enough to stand out from association studies like CVAS, the search for such a rare mutations, including *de novo* mutations, requires unbiased whole-genome or whole-exome sequencing. Most rare-variant studies have focused on the protein-coding parts of the human genome (the exome), thus allowing the detection of missense mutations, which are likely to be more penetrant. Moreover, the exome comprises only about 1.5% of the human genome, thus requiring less sequencing than the whole genome, and so larger number of patients can be sequenced within one study.

Rare-variant studies can be performed following two main approaches: **case-control** and **trios**. As in CVAS, case-control studies for rare mutation detection involve sequencing of a large number of affected versus unaffected individuals. On the other hand, the trio-based studies only focus on mutations present in child but not his parents (called *de novo* mutations). Most genomes contain about 77 new mutations not present in parent's genomes and usually about 0–2 of these new mutations are present in the exome, and about half of them will affect the sequence of an encoded protein. *De novo* mutations are now well studied and have been proved to play a prominent role in both rare (syndromic) and common (sporadic) forms of neuropsychiatric diseases such as ASC, Schizophrenia or intellectual disability (Fromer et al., 2014; Iossifov et al., 2014; McCarthy et al., 2014). At each generation, approximately 74 *de novo* SNVs, 3 indels and 0.02 CNVs arise in the sibling genome compared to his parents (Veltman en Brunner, 2012).

1.2.3 Network-based approach to unravel impacted biological pathways

Identification of disease-related genetic risk factors can be used for diagnostic purposes, especially for monogenic diseases. However, mutations by themselves are poorly informative on the underlying biology of pathogenesis and disease evolution. Rather, understanding in which biological process the proteins coded by the altered genes are engaged is of crucial importance to unravel the molecular basis of brain disorders. Several approaches can be instrumental to this purpose. The most frequently used are **protein-protein interaction (PPI)** (Gonzalez en Kann, 2012), **network-based analysis** (Cho et al., 2012) and **gene ontology** study (Shah et al., 2012), which are often performed altogether.

Proteins are constitutive of all organisms. In order to mediate metabolic and signalling pathways, and other cellular processes, proteins interact with other proteins, with RNA and /or with DNA. A mutation of the gene coding for a protein can have a dramatic deleterious effect on the protein functions (disruptions in protein-DNA interactions, protein misfoldings, new undesired interactions...). Therefore, mapping the physical PPI can provide a vast source of molecular information. Furthermore, PPI networks can elucidate the molecular basis of disease, which in turn can be useful for prevention, diagnosis, and treatment.

Practically, in PPI network (see section 1.4), the nodes represent the proteins and the lines connecting them represent the interactions between them. **Physical PPI** networks map the link of real protein interaction, detected through experimentation like co-immuno-precipitation, which is thus biased by an *a priori* knowledge. However, physical PPI network has evolved in more complex and integrated genotype-to-phenotype **functional networks**, less biased and more informative. Indeed, by assuming such a relationship

in polygenic brain disorders, not only are physical PPI between two proteins taken into account but also the probability that these two proteins share a genetic phenotype (e.g. shared GO annotations, shared biological pathways, shared tissue-specific expression, shared interaction partners or similar domain composition...). These functional network-based approaches, such as NetBag+ (see section 1.4, Figure 1.5), have been shown to be a powerful alternative approach to identify significant enrichment of specific molecular function, biological processes or cellular component (Cho et al., 2012).

In particular **Gene Ontology** (GO)-based computational tool like DAVID (Huang et al., 2009b) can be used to identify over-represented GO categories in a given gene set. This bioinformatics tool can of course be performed on the starting list of significantly altered genes from CVAS and RVAS neurogenomics data to have a first idea of the highest altered GO classes. Practically, these GO terms belong either to a biological process (BP), to a molecular function (MF), or to a cellular component (CC). The enrichment study of a particular GO category begins with two sets of data: the set of interest of size *M* and a reference set of size *N* (usually the whole human genome). Then, for a given GO category, we will have *m* genes of the set *M* belonging to this GO versus *n* genes of the set *N*. Enrichment of this GO is stated if, knowing that *n* genes among the *N* genes of reference are in this GO class, the occurrence of having *m* genes among the *M* genes of interest was not by chance (*p*-value less than 0.05, after multi-testing adjustment) (Shah et al., 2012).

1.2.4 Conclusion 1.2

In this section, we discussed about the polygenic architecture of mental disorders displaying various genetic risk factors that change the gene expression and function, and mentioned that network-based approaches and gene ontology classification can jointly unravel the relevant biological pathway impacted in each polygenic brain disease. These neurogenomics techniques made it possible to achieve major breakthrough, such as the discovery of potential impairment of neurobiological mechanisms like chromatin remodelling, transcription machinery or synaptic plasticity in Schizophrenia and ASC (McCarthy et al., 2014; O'Dushlaine et al., 2015; De Rubeis et al., 2014). However, how can a given microscopic genetic mutation induce a given macroscopic abnormal phenotype? These genotype-to-phenotype relationships are still poorly understood, and it is therefore of crucial importance to develop new tools to measure the functional impact of brain disease-related genetic risk factors.

1.3 Functional impact of synaptopathy-related genetic variants

Although key leaders in the field of psychiatry proposed a more integrated phenotype analysis based on connectomics (Akil et al., 2010), there are increasing genetic evidences that converge on specific molecular complexes, with postsynaptic components of excitatory synapses being the most important one in both Schizophrenia (Glausier en Lewis, 2013) and ASC (Peça et al., 2011), but also in Alzheimer's disease (Kirov et al., 2011; Penzes et al., 2011). To allow neurobiologists to get these so called synaptopathies under scrutiny (Ting et al., 2012), several powerful methodologies have been developed in many different fields, ranging from transgenic models to neurobiological techniques to microscopic approaches.

Therefore, we describe in this section two of the most powerful techniques standardly used to study the synaptic plasticity and which allow recent achievements in the understanding of the functional impact of psychiatric risk factors on postsynaptic excitatory synapses. First, intracellular and extracellular electrophysiological recording techniques and their recent applications in ASC and Schizophrenia are detailed. Then, super-resolution nanoscopy is briefly described followed by recent achievements in a context of brain diseases. Some personal results are shown for these two approaches that were performed for other projects from the CPN's group.

1.3.1 Electrophysiology and brain disorders

Electrophysiology techniques

Electrophysiology is the study of the electrical properties, applicable at different scale of biological systems ranging from single ion channel proteins to whole organs like brain. All the related techniques rely on the Ohm's law V=R.I. The measurements of the electric potential difference (V) or current (I) changes in neurons, and particularly action potential activity, have made it possible to understand fundamental neurobiological mechanisms. Moreover, they have shown to be invaluable approaches to evaluate the functional impact of psychiatric risk factors likely to induce electrophysiological abnormalities related to postsynaptic or pre-synaptic dysfunctions. Practically, electrical activity can be recorded from *in vivo* brain, acute brain slice or *in vitro* cultured neurons using either a solid conductors or a glass pipette filled with electrolyte solution. Mainly three different classical electrophysiology techniques exist depending on the electrode position (inside, upon or outside) with respect to the plasma membrane of the neurons, leading to either intracellular or extracellular recording of electrical activity. Here we only focus on intracellular recording using the whole-cell patch-clamp strategy (used in Chapter 2), and extracellular recording (used in this section).

One technique to record intracellular electrical activity is the **patch-clamp technique** (see Figure 2.15 in Chapter 2), which was developed by Erwin Neher and Bert Sakmann, rewarded by the Nobel Prize in 1991 (Neher en Sakmann, 1976; Sakmann en Neher, 1984). Contrary to conventional intracellular recording, which consists in perforating the neuron membrane with a sharp electrode, whole-cell patch-clamp recording takes a different approach. A patch-clamp microelectrode is a micropipette with a tip diameter of few micrometers, filled with electrolyte solution, the composition of which mimics the intraneuronal one. The microelectrode is slowly driven onto the cell membrane until the microelectrode tip attaches a piece of the cell membrane (the "patch"), thus showing a high resistance "sealing". By applying high pressure through mouth suction, the small patch of membrane in the electrode tip is cracked, thus allowing stable intracellular recording of electrical activity. Then, briefly, two different approaches of intracellular recording can be applied: voltage and current clamp. The former (the latter resp.) technique allows the operator to fix ("clamp") the cell potential (current

resp.) at a specific value and then subsequently record the current (voltage resp.), based on the Ohm's law. This makes it possible to measure how much ionic current crosses the plasma membrane at any given voltage, or how membrane voltage adjusts to a given current. We tested this approach to internalise fluorescent nanodiamonds in primary culture of hippocampal neurons, as described in Chapter 2.

Pioneered by David Hubel and Torsten Wiesel (Nobel Prize in Physiology or Medicine in 1981), extracellular recording consists in introducing an electrode into the brain of a living animal or into an acute brain slices and further detect electrical activity that is generated by the neurons close to the electrode tip. Not only is the spontaneous electrical activity being recorded, but also extracellular electric field can be recorded through electrical stimulation in a given brain sub-region (e.g. granular cells in dentate gyrus of hippocampus) which will synchronize many neurons displaying the same specific synaptic connection in another brain sub-region (e.g. CA3 pyramidal neurons through Mossy fiber synapses in the *Stratum Lucidum*; Figure 1.3). This approach is used in this section to study the functional impact of DYRK1A gene overexpression on NMDA-independent long-term potentiation (LTP) in Mossy Fiber synapses.

Eventually, due to limitations in studying larger spatial distribution of neuronal activity using classical techniques, electrophysiological activity monitoring can also be performed with light, with simultaneous optical perturbation and measurement of membrane voltage (Hochbaum et al., 2014). Optical recording of electric activity was thus developed through the well-known voltage-sensitive dyes, calcium sensitive fluorescent proteins and more recently under Karl Diesseroth's lead, engineered ion channels to allow tremendous achievements (Chen et al., 2013; Fenno et al., 2011; Ferezou et al., 2006)

Electrophysiology reveals functional impact of psychiatric risk factors

In the recent literature, the whole-cell patch-clamp intracellular recording and extracellular field potential recording are both broadly used to understand the functional impact of psychiatric risk factors and to evaluate drug treatments on animal models.

Neurexins constitute a well-known family of proteins impacted in ASC and Schizophrenia as reviewed in (Südhof, 2008). In human, neurexin genes *NRXN1*, *NRXN2* and *NRXN3* are known to code for trans-synaptic cell adhesion molecules that organize and synchronize assembly of synapses, as well as determine their spatiotemporal properties and diversity. Mutations in human *NRXN1* and *NRXN2* genes have been associated with autism and schizophrenia, while genetic variants in human *NRXN3* have been associated with autism, drug addiction and obesity. The group of Thomas Südhof's (Nobel Prize of Physiology or Medicine 2013), which is one of the most active in the world, tries to dissect the functional impact of these different risk factors. Due to its historical expertise, this group used electrophysiology approach extensively. Combining intracellular mEPSC and mIPSC (miniature Excitatory/Inhibitory PostSynaptic Current) and extracellular fEPSP (field Excitatory PostSynaptic Potential) recording with conditional or constitutive transgenic knock-in and knock-out mouse, they were able to address and answer very precise and specific neurobiological questions (see Chapter 4, Section 2 for details on the neurobiologists' toolbox). Consequently, they were able

to demonstrate that NRX3 performs distinct pre- or postsynaptic functions in different brain regions by distinct mechanisms, as well as the unexpected specific role of an alternative splicing of this protein. Indeed, extracellular part of presynaptic NRX3 protein mediates trans-synaptic regulation of postsynaptic AMPA receptors in the hippocampus, whereas in the olfactory bulb, intracellular part of presynaptic NRX3 protein mediates presynaptic GABA release (Aoto et al., 2013, 2015).

Another very recent example are from non-coding variants in the human *miR137* gene *locus* in which SNVs were associated to ASC and schizophrenia (Siegert et al., 2015). The authors dissect the functional consequence of four associated risk alleles. They performed fEPSP recording to study the functional impact of these genetic variants on the NMDA-independent LTP, as miR137 is enriched in the dentate gyrus. They proved that rare SNV of *miR137* caused down-regulation of the presynaptic target genes *CPLX1*, *NSF* and *SYT1*, leading to impaired vesicle release, and therefore impaired induction of Mossy fiber long-term potentiation and deficits in hippocampus-dependent learning and memory.

Eventually, electrophysiological approaches are also extremely powerful techniques to follow response to treatment and some researchers are working on the development of new drugs to cure or at least treat ASC. For example, one of the leader group in this field is the Yehezkel Ben Ari's group from INMED in Marseille (France). Combining electrophysiological recording and behavioral phenotyping, this group was able to prove that the administration of bumetanide –a diuretic drug– during the delivery process induces the release of oxytocin in pregnant mouse model of autism, which a rescue of the abnormal phenotypic traits. Conversely, when oxytocin is blocked in naïve pregnant mothers, offspring get autistic-like features. This discovery can lead to direct translational application to short circuit the autism pathogenesis in the offsprings during gestation (Tyzio et al., 2014).

Altogether, these three examples we highlighted stress how unique and powerful electrophysiological techniques are to unravel the functional impact of psychiatric-related genetic risk factors in primary neurons culture and acute brain slices.

Down Syndrome-related DYRK1A overexpression impairs synaptic plasticity

Aside from my PhD project, I contributed to another project of Pr. SIMONNEAU in collaboration with Dr. Valérie CRÉPEL (INMED, Marseille). The goal of this project (Viard et al., in preparation; see Appendices) is to understand whether or not *Dyrk1a* gene overexpression impacts NMDA-independent LTP in the context of Trisomy 21 (Down syndrome). *Dyrk1a* displays high levels of expression in dentate gyrus, CA1 and CA3 hippocampus subregions (Figure 1.3a). Therefore, we established transgenic mouse (called 189N3) with an extra copy of *Dyrk1a* gene, allowing to study the functional effects of a dosage of Dyrk1a similar to that found in Down syndrome afflicted individuals. We then performed fEPSP recording in the hippocampus to analyse the synaptic plasticity at the afferent Mossy fiber synapses in the *stratum lucidum* following electrical stimulation in the dentate gyrus (Figure 1.3b). We found that the non NMDA-dependent LTP was absent in the dentate gyrus-CA3 synapse (p = 0.0057) (Figure 1.3c). Material and methods are described in Appendices.



Figure 1.3: DYRK1A gene overexpression impacts non-NMDA dependent LTP at the dentate gyrus-CA3 hippocampal synapse. (a) False-color image of ISH from Allen Brain Atlas showing DYRK1A transcript expression in adult mouse hippocampus. Scale bar: $500 \mu m$. (b) Description of the Mossy Fiber synapses between DG (stimulation) and CA3 in the stratum lucidum (recording). (c) Summary data of mossy fiber field-EPSPs (fEPSPs) recording. Left: electrical field depression following stimulation before (black) and after (red) for WT and 189N3 TG acute slice. Middle: time course of the fEPSP amplitudes before and after mfLTP induction (black arrow) for three experiments. Right: data are means \pm SEM and calculated from 4 different mice with 3 slices per mouse for both genotypes. ** corresponds to p<0.01.

1.3.2 Super-Resolution nanoscopy and brain disorders

Super-Resolution techniques

In addition to electrophysiology, one of the most successful techniques to evaluate the functional impact of synaptopathy-related genetic risk factors is the fluorescence microscopy. Indeed, it is commonly used to study dynamic processes inside living cells with exquisite sensitivity and specificity. More precisely, super-resolution fluorescence microscopies are techniques with resolution better than the diffraction limit. They allow to perform a structural analysis of subcellular entities and macromolecular complexes having sizes of only a few tens of nanometres.

New super-resolution techniques split into two main groups. The first group encompasses techniques that exploit non-linear response of the fluorescence under patterned light excitation. Stimulated emission depletion microscopy (STED) (Hell en Wichmann, 1994; Willig et al., 2006) and structured illumination microscopy (SIM) (Gustafsson, 2000, 2005) belong to this category. The second group includes techniques that are based on randomized single-molecule localization, namely photo-activated localization microscopy (PALM)/stochastic optical reconstruction microscopy (STORM) (Betzig, 1995; Betzig et al., 2006; Rust et al., 2006). Other closely related techniques, such as RESOLFT, sptPALM or uPAINT, have been developed and further used in neurosciences, leading to tremendous breakthrough. As these techniques are out of the scope of this PhD manuscript, their description and specific applications in neurosciences will not be provided here and readers are invited to refer to the following reviews (Hell, 2007; Huang et al., 2009a; Maglione en Sigrist, 2013; Tønnesen en Nägerl, 2013).

However, as we will apply dSTORM super-resolution nanoscopy in the last part of this subsection, a brief description of this technique is given hereafter. Direct stochastic optical reconstruction microscopy (dSTORM) uses conventional fluorescent probes and enable imaging of cellular nanostructure with a lateral resolution of $\sim 20/30$ nm. In contrast to PALM, dSTORM experiments start with fluorescent probes being in the bright ON state and will immediately be transferred to a metastable and reversible OFF state, with a lifetime ranging from 100 ms to several seconds. Then, a sparse subset of fluorophores is getting back to the ON state, either spontaneously or by photoinduction through irradiation with a second laser at 405 nm wavelength. The fluorescence is detected using a wide-field fluorescence microscope equipped with a sensitive CCD array detector and the centroid coordinates of single molecule spots are precisely and accurately determined. Repetitive activation / localization process allows a temporal separation of spatially unresolved structures and a super-resolution image is finally reconstructed using all single-molecule localizations (Figure 1.4a). When using a localisation-based super-resolution approach, it is important to minimize artefacts in data analysis, thus being sure that only one single fluorophore emits at each frame within a diffraction-limited area (van de Linde et al., 2011). As an example, a major breakthrough in the mechanism of chromatin remodelling has been recently achieved using dSTORM super-resolution microscopy (Ricci et al., 2015).

Super-resolution nanoscopy reveals some functional impact of psychiatric risk factors

The combination of super-resolution techniques with human genetics and transgenic mouse model very recently appeared to be a powerful approach to elucidate gene function in health and disease, and therefore to understand the functional impact of genetic variants.

The first example came from STED nanoscopy that was used to reveal nanoscale defects in the dendritic spine morphogenesis in a mouse model of Fragile X Syndrome (FXS) (Wijetunge et al., 2014). Related to ASC, FXS is associated with impairment of the human gene *FMR1* (Fragile X Mental Retardation 1) located on the X chromosome. This human gene codes for the FMRP protein. By mating *FMR1*-KO mouse with *YFP*-KI, the authors obtained volume intraneuronal staining and used STED to precisely image the dendritic spines in acute brain slices. Surprisingly, they found that the spine morphogenesis in *FMR1*-KO mice was not impacted, except for age-dependent and brain region-dependent alterations, which are only detectable at the nanoscale.

Another example came from SIM super-resolution microscopy. Common and rare variants in the *ANK3* gene, encoding ankyrin-G protein, have been associated with bipolar disorder, schizophrenia, and ASC. This protein is a scaffolding adaptor that links membrane proteins to the actin/spectrin cytoskeleton and organise them into specific domains in the plasma membrane. Using SIM super-resolution microscopy, Smith et al. found that ankyrin-G forms distinct domain nanostructures within the spine head and neck (Smith et al., 2014). Therefore, these results bridge the gap between a putative post-synaptic disease-associated gene and a functional key role in dendritic spine sub-compartment at the nanoscale, opening directions for basic and translational investigation.

Super-resolution microscopy is also an extremely powerful technique to follow response to drug treatment. For example, by using STORM approach, Dudok et al. demonstrated that chronic THC administration induces endocannabinoid receptor (CB1) downregulation in a dose-dependent manner (Dudok et al., 2014). CB1 receptors are known to mediate the cannabis effects in humans, with strong and long-lasting cognitive deficits for chronic users. Practically, to unravel the nanoscale organization of CB1 in a cell type– and subcellular compartment–specific manner, the authors combined electrophysiology and confocal morphology analysis with high-throughput STORM microscopy on acute brain slice to characterize CB1-positive synapses.

Altogether, the three examples we highlighted here stress how unique and powerful super-resolution techniques are to unravel nanoscale organisation of protein complex and the functional impact of psychiatric-related genetic risk factors in primary neurons culture and acute brain slices.

AUTS2 is a key regulator of synaptic plasticity

Aside from my PhD project, I contributed to another project of Pr. SIMONNEAU in collaboration with Antoine TRILLER's group (IBENS, Paris). The goal of this project (Lepagnol-bestel et al., in preparation; see Appendices) is to decipher the role of *Auts2* in synaptic plasticity through its direct action as a postsynaptic protein. *Auts2* is a key gene with numerous mutations found in Autism and Schizophrenia (Oksenberg en Ahituv, 2013) (see Chapter 4, section 4.4 for more genetic details). *Auts2* was recently demonstrated to be located either in the nucleus where it plays the role of a transcriptional and chromatin regulator (Gao et al., 2014), or in the growth cones of early-stage developing neurons with an unknown function (Hori et al., 2014). Here, we decided to use super-resolution nanoscopy to precisely localized Auts2 proteins inside the dendritic spines (Figure 1.4). Auts2 clusters were found to be both in the dendritic shaft and spines. Half of the dendritic spines have clusters only inside the spine head, 16% only in the neck, 26% in both neck and head and only 10% of the dendritic spines show no Auts2 clusters. Moreover, the number of Auts2 clusters per dendritic spines was measured to be around 4 in the head or neck and 8 in the entire dendritic spine. We also found linear correlations of the number of Auts2 clusters with respect to the spine area, the spine length and the head width (Figure 1.4f-h). Eventually, we observed that the sub-compartmentalization of Auts2 clusters is dependent of the synaptopodyne protein (Figure 1.4i-k).



Figure 1.4: Superresolution dSTORM imaging of Auts2 clusters inside dendritic spines. (a-c) Subcompartmentalization of Auts2 clusters inside dendritic spines as shown by dSTROM imaging of Auts2-Alexa647 (a, top) merged with conventional imaging of actin stained with phalloïdine-Alexa488 (a, bottom and b). Scale bar: 1µm. (c) Auts2 clusters were found to be both in the dendritic shaft and spines. Half of the dendritic spines have clusters only inside the spine head (green), 16% only in the neck (salmon), 26% in both neck and head (purple) and only 10% of the dendritic spines show no Auts2 clusters (blue). (d) Histogram of the Auts2 cluster per dendritic spines, with in average 4,1±0.3 clusters/spine. (e) Cumulative probability density of the number of Auts2 cluster in neck (salmon), neck&head (purple) and head (green). Inset: bar plots show the average of Auts2 cluster per spine sub-region, with 3.6±0.2 clusters in the head, 3.5±0.5 clusters in the neck and 7.1±0.6 clusters in the entire spine (neck&head). (f-h) Linear correlations of number of Auts2 clusters with respect to spine area (f), spine length (g) and head width (h). (i-k) The sub-compartmentalization of Auts2 clusters is dependent of synaptopodyne. (i) Conventional imaging of Actin (green), synaptopodyne (red) and super-resolution dSTORM image of Auts2 (white) and merged image showing that Auts2 clusters are preferentially localized in the neck for synpo-positive spines and in the head for synpo-negative spines. (j) Proportion of dendritic spines displaying Auts2 cluster only inside the spine head (green), only inside the neck (salmon), in both neck and head (purple) or with no Auts2 clusters (blue) for dendritic spines without (top) or with (bottom) synpatopodyne. (k) Quantification of the number of Auts2 clusters for each of the previous classes. All data are given as mean±s.e.m.;

figures inside bar plots in (e) represent the number of sample. Statistical significance was calculated using the Mann-Whitney test (***: p<0.001). Scale bars: $1\mu m$

1.3.3 Conclusion 1.3

Thanks to neurogenomics analysis of common brain diseases, a large repertoire of psychiatric risk factors has been catalogued, combining common and rare genetic variants. We briefly described two powerful approaches standardly used to gain insight into the functional impact of synaptopathy-related genetic variants, namely electrophysiology and super-resolution microscopy.

However, synaptic plasticity is not the only biological pathway which is impaired in neuropsychiatric and neurodegenerative diseases. Several sparse evidences point out abnormalities of the intraneuronal transport associated to brain diseases (Hirokawa et al., 2010; Millecamps en Julien, 2013), and one can wonder if this is a random observation or a generic rule.

1.4 Disease relevance of monitoring the intraneuronal transport

As discussed in the last part of section 1.2, one strategy to unravel which biological pathways are impacted in a given brain disease consists in combining functional network biology approach with gene ontology (GO) classification (Cho et al., 2012; Gonzalez en Kann, 2012; Shah et al., 2012). Several networkbased approaches have been developed so far but NETwork-Based Analysis of Genetic associations (NETBAG) developed in 2011 by the Vitkup's group proved to be the most relevant. Therefore, in this section, we briefly describe NETBAG+ and subsequently apply this method to a bioinformatics meta-analysis of schizophrenia (SZ) gene datasets, followed by a GO enrichment analysis of the established network. Finally, we apply GO enrichment analysis to *de novo* variant datasets of SZ and ASC. All bioinformatics analysis described in this part were performed by Yann LOE-MIE, PhD.

1.4.1 Brief description of the NETBAG+ network-based approach

As mentioned in section 1.2, geneticists developed the network-based approaches to understand which biological pathways are impaired in a given brain disease (Figure 1.5 and Figure 1.6). The specificity of NETBAG+ is that it assumes a genotype-to-phenotype relationship. Therefore, it is a more complex and integrated **functional network**, than standard physical PPI networks. Here, each edge of the network is scored given the probability that the corresponding pair of genes is involved in the same genetic phenotype (e.g. shared GO annotations, shared biological pathways, shared interaction partners, shared tissue-specific expression or similar domain composition...). Usually, edge widths are proportional to the likelihood score between the two genes (Figure 1.5a), and node sizes are proportional to the gene contribution to the sub-network (e.g. hub genes with many connections like HTT or MYH9 in Figure 1.6). Such a phenotype network is established based on all genes in the human genome (control genes) (Figure 1.5a). Then, for a given disease, different types of genetic variations (CNV blue; SNV green; GWAS red) are mapped on this network (Figure

1.5b). Finally, sub-network with the highest additive score is selected (black circle) and the statistical significance of this result is measured by a randomization test (Chang et al., 2015; Gilman et al., 2011, 2012).



Figure 1.5: The NETBAG+ functional approach for gene cluster identification. (a) A phenotype network is established based on all genes in the human genome. From (Gilman et al., 2011). (b) For a given brain disease, different types of genetic variations are mapped on this network (CNV blue; SNV green; GWAS red). From (Gilman et al., 2012).

Because several genes are included in one CNV region, the NETBAG method was historically applied to identify a biological network affected by CNVs in ASC (Gilman et al., 2011). Interestingly, they found that the GO "establishment and maintenance of cell polarity" is ranked in the top 10 enriched GO for this disease. This biological process is well-known to be regulated by microtubule organisation which is molecular motordependent (Kapitein en Hoogenraad, 2015). Then, this approach was extended (NETBAG+) to take into account all possible genetic variants (SNV, CNV, GWAS) and was applied to schizophrenia (Gilman et al., 2012). At this time, the initial gene dataset was smaller than the one we use hereafter.

1.4.2 Application of NETBAG+ to a gene dataset of Schizophrenia

To elucidate functional networks perturbed in schizophrenia, we applied NETBAG+ computational method to a set of genes affected by *de novo* SNVs (n = 609) (Fromer et al., 2014) and CNVs (n = 58) (Rees et al., 2014b). We also used GWAS data from a multi-stage schizophrenia genome-wide association study involving up to 36,989 cases and 113,075 controls (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). This study identified 108 *loci* from which 347 genes were found associated to schizophrenia. From this repertoire of 1014 altered genes, we found that the largest network comprises 559 genes (p = 0.012) (see Appendices). We used DAVID methodology (Huang et al., 2009b) to identify Gene ontology terms that are significantly enriched among network gene annotations (Table 1.1). We first observed an alternation between transport-related and synapse-related gene ontology (in red and green respectively in Table 1.1).

N	X	\pmb{P}_{adj}	GO identifier	Ontology	GO term
25	253	0.003	GO:0007017	BP	microtubule-based process
24	298	0.03	GO:0007268	BP	synaptic transmission
23	269	0.002	GO:0015629	CC	actin cytoskeleton
23	244	0.01	GO:0000904	BP	cell morphogenesis involved in differentiation
22	245	0.016	GO:0048858	BP	cell projection morphogenesis
21	246	0.004	GO:0044456	CC	synapse part
21	274	0.009	GO:0005874	CC	microtubule
19	141	< 0.001	GO:0008022	MF	protein C-terminus binding
19	209	0.034	GO:0048667	BP	cell morphogenesis involved in neuron differentiation
19	213	0.040	GO:0048812	BP	neuron projection morphogenesis
19	220	0.048	GO:0007067	BP	mitosis
19	220	0.048	GO:0000280	BP	nuclear division
18	205	0.007	GO:0005635	CC	nuclear envelope
18	224	0.013	GO:0005813	CC	centrosome
18	253	0.037	GO:0005815	CC	microtubule organizing center
16	113	< 0.001	GO:0044449	CC	contractile fiber part
16	142	0.007	GO:0003774	MF	motor activity
16	163	0.046	GO:0043062	BP	extracellular structure organization
15	147	0.007	GO:0005819	CC	spindle
15	140	0.017	GO:0005516	MF	calmodulin binding
14	111	0.003	GO:0030016	CC	myofibril
14	135	0.007	GO:0045211	CC	postsynaptic membrane
13	98	0.002	GO:0030017	CC	sarcomere
13	113	0.045	GO:0007018	BP	microtubule-based movement
12	71	0.001	GO:0014069	CC	postsynaptic density
11	61	0.009	GO:0050808	BP	synapse organization
11	95	0.014	GO:0046930	CC	pore complex
11	77	0.016	GO:0003777	MF	microtubule motor activity

Table 1.1: Alternation between transport-related and synapse-related GO terms significantly enriched in the NETBAG+ functional network (see Appendices). GO enrichment analysis of the 559 genes was performed with DAVID (Huang et al., 2009b). N is the number of network genes annotated with a given GO term and X is the total number of human genes in the same GO. p_adj values in the table represent p-values adjusted by Benjamini-Hochberg procedure in DAVID. MF: molecular function; BP: biological process; CC: cellular component.

The three most significant GO term are Microtubule-Based Process (MBP), Actin Cytoskeleton and Synaptic Transmission, whose altered gene for each of them are shown in Table 1.2. When considering only these genes for the network mapping of NETBAG+ (Figure 1.5b), we obtained the sub-network shown in Figure 1.6.

Microtubule-Based Process (GO:0007017)	Synaptic Transmission (GO:0007268)	Actin Cytoskeleton (GO:0015629)
CLASP1 (none)	AKAP9 (SNV)	ACTA2 (SNV)
CTTNBP2 (SNV)	ALDH5A1 (SNV)	CAPZA1 (SNV)
DCTN3 (SNV)	BAIAP3 (SNV)	CTNNA2 (SNV)
DNAH1 (SNV)	CACNA1C (GWAS)	DBN1 (SNV)
DNAH3 (SNV)	CACNB2 (GWAS)	DCTN3 (SNV)
DNAH9 (SNV)	CTNNA2 (SNV)	EPB41 (SNV)
HAUS6 (SNV)	CTTNBP2 (SNV)	ESPN (SNV)
HAUS8 (SNV)	DMD (SNV)	KLHL20 (SNV)
HTT (SNV)	DRD2 (GWAS)	MYH11 (SNV)
KIF13B (SNV)	DVL1 (SNV)	MYH9 (SNV)
KIF14 (SNV)	GABRD (CNV)	MYO15A (GWAS&SNV)
KIF18A (SNV)	GRID2 (SNV)	MYO18A (SNV)
KIF1A (CNV&SNV)	GRIN2A (GWAS&SNV)	MYO18B (SNV)
KIF20B (SNV)	GRM3 (GWAS)	MYOZ2 (SNV)
KIF3A (SNV)	HTR2A (SNV)	NEB (SNV)
KIF6 (SNV)	KCNQ5 (SNV)	PRKCZ (CNV)
KLC1 (GWAS)	LPAR3 (SNV)	SHROOM1 (SNV)
MARK4 (SNV)	NRXN1 (SNV)	SLC9A3R1 (SNV)
MYH9 (SNV)	PTPRF (GWAS&SNV)	SORBS2 (SNV)
PCNT (SNV)	RIMS1 (SNV)	SRCIN1 (SNV)
PEX1 (SNV)	SLC1A1 (CNV)	STK38L (SNV)
PRKCZ (CNV)	SLC5A7 (SNV)	TTN (SNV)
TNKS (SNV)	SLC6A4 (none)	UTRN (SNV)
TTK (SNV)	VDAC1 (SNV)	
UBE2C (SNV)		

Table 1.2: Genes of the network associated to the three first GO categories from Table 1.1. Type of variants (SNV, CNV or GWAS) are indicated in parenthesis.

The GO MBP contains genes encoding proteins that are associated with intraneuronal transport such as MARK4 which phosphorylates microtubule-associated proteins, KIF1A, KIF13B or KIF20B which are members of kinesin family, DNAH1, DNAH3 and DNAH9 which are members of dynein family and *DCTN3*, another member of DCTN protein family essential for dynein activity *in vivo* (McKenney et al., 2014). Here, seven *KIF* genes are part of the 559 genes network, indicating a statistically significant enrichment (p < 0.0001) for the KIF superfamily that includes 45 genes (http://www.genenames.org/genefamilies/KIF). The actin subcluster (GO: 00015629; Actin Cytoskeleton) contains actin-related proteins (ACTA2, CAPZA1, CTNNA2) and myosins (MYO15A, MYO18A or MYO18B). The synapse sub-cluster (GO: 0007268; Synaptic Transmission) contained synaptic adhesion molecules such as neurexin (NRXN1), components of the presynapse (RIMS1), receptors of glutamatergic synapses (GRM3, GRIN2A, GRID2), AKAP9 that directly interacts with NMDA receptors and voltage-gated calcium channels (CACNA1C, CACNB2). It also includes the dopaminergic receptor (DRD2) that is the target of all effective antipsychotic drugs, as already mentioned the General Introduction of this PhD Manuscript.



Figure 1.6: The NETBAG+ functional sub-network of genes belonging to the three highly enriched GO classes (*described in table 1.1 and 1.2*).

1.4.3 Intraneuronal transport as a relevant endophenotype of ASC.

The genetic overlapping of mental disorders such as SZ, ASC or intellectual disability (ID) was recently reported. In 2013, the genetic relationship between five major psychiatric disorders, from which ASC and SZ, was demonstrated using CVAS gene sets (Group en Consortium, 2013). Interestingly, they showed significant overlaps between SZ and ASC. Then, in 2014 and 2015, studies reported overlapping between SZ, ASC and ID *de novo* mutation gene sets (Fromer et al., 2014; Li et al., 2015).

We therefore evaluated the genetic overlapping between the GO MBP category previously studied, and SZ, ASC and ID *de novo* mutation gene sets, as for (Fromer et al., 2014). Using Babelomics v4.2 (Medina et al., 2010), we found statistically significant overlapping of the MBP GO term with the ASC and SZ-related *de novo* mutation repertoires (p = 0.00049 and p = 0.00013 respectively), as shown in the bar plot of Figure 1.7. Conversely, no overlapping was found between MBP GO category and IDs *de novo* mutation repertoires.

Altogether, these findings suggest that microtubule-based processes impairment may result in abnormal endophenotype for both schizophrenia and autism.



Figure 1.7: Enrichment of Gene Ontology "Microtubule-Based Process" in Schizophrenia (SZ) and in Autism Spectrum Condition (ASC). In Venn diagram (left), the overlap area indicates the number of genes between/among different disorders and MBP.

1.4.4 Conclusion 1.4

In this section, we showed that the NETBAG+ network-based methodology combined with gene ontology classification is a powerful approach to unravel biological pathways impacted in common polygenic mental disorders. By applying it to a meta-analysis data set in schizophrenia, we demonstrated the enrichment of the Microtubule-Based Process GO category. Moreover, we also obtained enrichment of this MPB GO class for ASC gene data set, and subsequent overlapping between schizophrenia and ASC, as already reported elsewhere. Altogether, for the first time, we showed that intraneuronal transport is a relevant endophenotype of neuropsychiatric diseases. Our study illustrates the complex and evolving view of the polygenic architecture of psychiatric diseases and suggests a possible change from synapse to transport paradigm.

1.5 Conclusion of Chapter 1

In this chapter, we briefly drew up the state-of-the-art of mental disorders genetic risk factors. We showed that the clinical phenotypes of these diseases are associated to genotype enrichment of mutated genes. Among them, genes related to synaptic function are found, with still the open question of whether dendritic spine impairment is the cause or the consequence of the neuropathology. Well-established and new techniques help to unravel abnormal endophenotypes in synaptic plasticity. Electrophysiology, super-resolution microscopy and optogenetics just started to give novel insights into the nanoscale organization and the dynamic of chromatin remodeling (Ricci et al., 2015), transcription machinery (Chen et al., 2014; Normanno et al., 2015) and synaptic plasticity (Dudok et al., 2014; Smith et al., 2014).

However, several genetic and functional neurobiology studies recently pointed out the role of abnormal intraneuronal transport of proteins in neuropsychiatric diseases (Hirokawa et al., 2010; Kitagishi et al., 2015; Millecamps en Julien, 2013). Several protein kinases regulating the membrane trafficking have been shown to be involved in ASC (Kitagishi et al., 2015). Using a bioinformatics meta-analysis of recent schizophrenia and ASC gene datasets, we found an enrichment of altered genes coding for proteins involved in the intraneuronal transport. This neurobiological mechanism is of utmost importance for the establishment and maintenance of sub-neuronal compartments, such as synapses, because it delivers important materials to this specific locations far from the cell body (Figure 1.8).

The current paradigm in the field is that patients with mental disorders display abnormalities of the synaptic function. However, because the intraneuronal transport is upstream to the synaptic function, our hypothesis is that abnormalities of the intraneuronal transport might induced endophenotypes of mental disorders. Therefore, the same way as electrophysiology can be instrumental to detect subtle abnormalities of the synaptic plasticity, we now have to develop a methodology that can be instrumental to detect slight impairment of the intraneuronal transport.



Figure 1.8: A possible change of paradigm in the field of brain diseases: from synaptic defect to transport defect.
CHAPITER 2

Fluorescent Nanodiamonds: toward non-genetic nano-reporter of intraneuronal transport dynamics

Summary

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2.1 Introduction

From the Chapter 1, we know that brain diseases are based on polygenic risk factors. Then, we know that numerous impacted genes are engaged in specific biological processes such as chromatin remodelling or synaptic plasticity. In the last section of this chapter, we have showed that brain disease-related genetic risk

factors may induce abnormalities in microtubule-based transport and can therefore be a relevant biomarker of ASC and Schizophrenia. Overall, these observations prompted us to mimic *in vivo* the subtle gene expression changes linked to brain disease-related genetic variants and subsequently quantify their functional impact on the intraneuronal transport using primary cultures of hippocampal neurons.

To this aim, the use of fluorescent probes seem to be the appropriate tool as they are powerful and versatile tools for *in vivo* and *in vitro* spatiotemporal study of complex biological mechanisms. However, given the plethora of genetically and non-genetically encoded nano-sources of light, the optimal approach to address the aforementioned question has to meet several criteria: (i) keep the homeostasis integrity of neurons (i.e. no genetic modification; no cytotoxicity); (ii) allow long-term bioimaging in neurons (i.e. perfect photostability, no cytotoxicity); (iii) reach high spatiotemporal resolution (i.e. high brightness and large signal-to-background ratio, perfect photostability); (iv) produce high-throughput data (i.e. reproducibility and efficiency of the method).

In this second chapter, we first detail the different types of fluorophore suitable for intracellular dynamical studies. Then the production and physical properties of fNDs are described, along with some biological applications. Finally, the different strategies tested to internalize fNDs inside neurons are discussed, as well as the wide-field microscopy technique used to perform single-particle tracking. We conclude this chapter with the qualitative description of the intraneuronal motions, validating our experimental pipeline

2.2 Fluorescent probes for intracellular transport recording

The usual tools employed to study the intracellular transport are based on nano-source of light. These nanotools rely on the fluorescence phenomenon, first describe in the late 16th century by Nicolás Monardes, a Spanish physician and botanist, and refined by George Gabriel Stokes in 1852 who created the term of "fluorescence" (fluo-*rite* and *opal*-escence). From organic fluorescent probes (e.g. fusion protein GFP family; synthetic dyes: Cy3, Alexa488) to solid-state inorganic fluorescent probes (e.g. fNDs, Qdot), a plethora of fluorescent probes can be used to dissect biological processes (Figure 2.1a). These probes are standard tools in all biology laboratories around the world, and they are becoming even more essential with super-resolution microscopy. Indeed, in Ernst Abbe's day and until the achievement of super-resolution techniques, better images were obtained with better microscope objectives (i.e. less aberrant and with larger numerical aperture), but today better images come from improvement of fluorophores photophysical properties (Marx, 2013). Therefore, it is important to be aware of all available probes and of their main physical characteristics, to be sure that we use them in a good manner (see Appendices).



Figure 2.1: Florescent probes and in vivo imaging. (a) Relative size of commonly used organic and inorganic fluorescent probes, with IgG antibody for the comparison. (from <u>http://zeiss-campus.magnet.fsu.edu/</u>). <i>(b) The NIR window is ideally suited for in vivo imaging because of minimal light absorption by haemoglobin (650 nm). Reprinted from (Weissleder, 2001).

2.2.1 Organic fluorescent probes

Organic fluorescent probes are the most widespread probes for fluorescence microscopy in biology. They can be "coupled" to biomolecules (peptides, proteins, oligonucleotides) or to cell compartments in three main ways: (i) by a covalent coupling (e.g. EDC/NHS amidation, click chemistry, halo-tag...); (ii) by affinity coupling (e.g. two conjugated antibody, streptavidin-biotin...); (iii) by producing *in vivo* an exogenous fluorescent protein (e.g. transfection, transduction...). The techniques of ImmunoCytoChemistry (ICC), transfection and transduction with adeno-associated virus (AAV) are described in Chapter 4, section 4.2 and Appendices, as we used them for this work.

Synthetic organic dyes such as Cyanine (Cy3, Cy5...) or Alexa-dye (Alexa488, Alexa 647...) are widely used for immunostaining. As shown in Figure 2.1a, they are small in size (few nanometers), and they present good performances (brightness, stability...) for super-resolution microscopy (dSTORM or STED). Apart from the organic dye molecules, the genetically encoded fluorescent proteins constitute another category of organic fluorophores widely used for bio-imaging. The Green Fluorescent Protein (GFP), originating from jellyfish, was the first fluorescent protein to be discovered. It was in 1962 when Osamu Shimomura purified it from the *Aequorea Victoria jellyfish* (Shimomura et al., 1962). GFP protein emits green fluorescence light under blue light excitation. It was only in 1992 that Douglas Prasher cloned the sequence of GFP and Martin Chalfie expressed this sequence *in vivo* (Prasher et al., 1992). Later on, the group of Roger Tsien reported the first crystal structure of GFP, showing the way for the creation of GFP mutants, to finally obtain different color variants and improve the fluorescence signal and photostability (Ormö et al., 1996; Shaner et al., 2005; Zhang et al., 2002). Shimomura, Chalfie and Tsien were awarded in 2008 with the Nobel Prize in Chemistry for their work on genetically encoded fluorescent proteins. The reason why GFP became so popular in biology is that

with no external labelling, cellular compartments, whole cells, tissues or organisms can be labelled using the appropriate GFP-cDNA.

Some drawbacks such as photobleaching and cytotoxicity arise when using organic fluorophore. Although methods have been developed to reduce these effects (by a weak excitation power, or by using oxygen scavenger systems), they still exist and makes organic dyes not the appropriate fluorescence probes for long-time observations of dynamic biological processes such as intracellular transport. Moreover, the use of chimeric protein-fluorophore constructs is limited in neuronal studies for some reasons (low transfection efficiency, uncontrolled protein overproduction and fluorescent protein photobleaching), to which we have to add toxicity for the transfected cells due to both the technique (lipofectamine induced depolarization of neurons) and the protein overexpression (usually, transfection has to be done early in hippocampal neurons development to limit mortality). Another possibility is to use knock-in transgenic mouse construct to control a 1:1 stoichiometry (see Chapter 4 section 4.2).

Nevertheless, organic fluorescent probes have been successfully used to study intracellular transport. We will present some recent examples but because transfection coupled to standard fluorescence microscopy is a very common way to study intraneuronal transport, we will only focus on super-resolution and optogenetic approaches. "Pointillism" super-resolution techniques STORM and PALM were shown to be powerful techniques to unravel new neurobiological mechanisms (Xu et al., 2013). However; the former approach does not allow dynamic live cell imaging and to our knowledge, the latter approach was never applied to study the intraneuronal transport dynamics (Hoze et al., 2012; Nair et al., 2013). Otherwise, even though STED super-resolution technique is based on raster scan, it was shown to be a powerful technique to dissect synaptic vesicle movement at high frame rate (Westphal et al., 2008). However, STED is not very suited to quantitatively study the intraneuronal transport in a high-throughput manner as it is subject to low transfection rate, photobleaching (high laser power needed) and small region raster scanning.



Figure 2.3: Local and reversible activation of microtubule-based transport with light. (*Left*) *Assay and constructs.* (*Right*) *Peroxisome distribution in primary hippocampal neuron expressing PEX–LOV and MYO–PDZ when activated with light.* Arrow heads point out spine with myosin. Scale bars, 5 µm. From (Bergeijk et al., 2015).

Another approach to investigate intraneuronal transport is the one developed first in Kapitein's group, and soon after in Cui's group (Bergeijk et al., 2015; Duan et al., 2015). Using dedicated genetic constructs and optogenetics, they succeeded in controlling and positioning organelles and molecular motors with light. The former group achieved even more impressive breakthrough by positioning one organelle inside a dendritic spines (Figure 2.3left). However, while our goal is to monitor the intraneuronal transport, this strategy aims at dissecting some fundamental transport mechanism (e.g. traffic laws, road map...) with optical manipulation of this process. Moreover, this approach is based on multiple transfections, leading to cytotoxicity and low throughput. But for sure, this optogenetic approach coupled with super-resolution microscopy, electrophysiology (Novak et al., 2013) and CRISPR/Cas9 genome engineering (Cong et al., 2013; Ratz et al., 2015) will lead to breakthroughs in a close future.

2.2.2 Inorganic fluorescent probes

Even though fluorescent organic probes can lead to unprecedented achievements in the understanding of intracellular mechanisms, photobleaching and cytotoxicity of overexpressed chimeric proteins or synthetic dyes make them inappropriate for long lasting live cell imaging. During the last 20 years, inorganic fluorescent probes have been proposed as an alternative. However, the application of nanoparticles as fluorescent probes in biology requires some specific properties:

• <u>Small size</u>: nanoparticles are highly mobile even in the viscous cellular environment. Additionally, for biomolecule tracking, the fluorescent probe must have a tiny size in comparison to the stained biomolecule, so that it does not impact its motion.

• <u>High brightness</u>: as the cytoplasm contains auto-fluorescent proteins (e.g. NADPH and flavins), fluorescent nanoprobes have to be a strong emitter to achieve high signal-to-background ratio. Moreover, for *in vivo* imaging, these probes should emit in the far red region (figure 2.1b)

• **Biocompatibility**: no toxicity for the cell and/or the organism, and should be easily degraded or eliminated for theranostic applications.

• <u>Well-defined surface chemistry</u> for further functionalization with biomolecules.

The most famous inorganic fluorescent particles are the semiconductor nanocrystals, so-called **Quantum Dots (QDs)** (Alivisatos, 2004), which are mostly composed of elements of the II/VI or III/V periodic table columns. Owing to their unique photophysical and photochemical properties, group II–VI (e.g. CdSe, CdS and CdTe) and III–V QDs (e.g. InAs/GaAs) have been widely used for many years to tackle biological questions both *in vitro* and *in vivo* (Biermann et al., 2014; Dahan et al., 2003; Gao et al., 2004).

These nanocrystals are made of a few hundreds to thousands of atoms and the electron wave function is confined by potential barriers in the three dimensions, forming a "point-like" structure. QDots photoluminescence characteristics stem from the small size of these particles leading to quantization of its energy levels making it possible to tune its emission wavelength (Alivisatos, 1996). The core size of typical QDs is 2-10 nm and a shell of ZnS is necessary to stabilize the fluorescence properties (Figure 2.4a). Polymer coating of QDots is then mandatory to obtain stable colloidal suspensions and suitable chemical groups for further bioconjugation. All in all, the total size of the particles is in the range of 20-50 nm which is one order of magnitude larger than a single dye molecule. However, the advantages of QDots over organic dyes are that they have a brighter fluorescence signal, thanks to a higher absorption cross section, and better photostability, thus allowing observations and tracking over an extended period of time.

However, QDs present also some drawbacks for biological applications, namely cytotoxicity and photoblinking. One of the main drawbacks of QDots for long-lasting bioimaging is the toxicity of its constitutive elements such as cadmium (Kirchner et al., 2005; Lewinski et al., 2008). The second drawback is related to photoluminescence binary state, alternating between bright and dark periods (Nirmal et al., 1996), which prevents from high fidelity single particle tracking (Figure 2.4). Yet, the blinking phenomenon can be reduced or even suppressed but at the expense of a higher photobleaching (Wang et al., 2009) or an increase of shell thickness (Mahler et al., 2008) (NB: Wang et al., 2009 was just retracted from Nature in November 2015 because of artefacts). Even though, one can also take advantage of this phenomenon to distinguish single dots from aggregates, and actually single QDots tracking has led to tremendous breakthroughs in neurobiology with the study of extracellular post-synaptic receptor trafficking (Choquet en Triller, 2013).



Figure 2.4: Structure and properties of QDots probes. (a) Schematic representation of a QDots probe. The inorganic CdSe core nanocrystal and ZnS shell (red) dictate the optical properties in a size-dependent manner. Organic surface coatings (grey) such as small molecules, peptides and amphiphilic micelles or polymers provide colloidal stability in biological buffers. Key features of the surface coating include PEG to reduce nonspecific interactions and reactive groups to enable conjugation of biomolecules. Biomolecules such as DNA, streptavidin and antibodies are conjugated to the surface to enable specific binding to biological targets. GFP and fluorescein dye are shown for size comparison. Scale bar, 5 nm. (b) Typical fluorescence images of a single QDots as observed by fluorescence microscopy showing changes in emission intensity (AU, arbitrary units). The intensity time trace illustrates the random alternation between 'on' and 'off' states, known as photoblinking, which is a signature feature of an individual QDots. From (Pinaud et al., 2010)

Application of QDots for intracellular transport study

QDots have been successfully used not only to probe extracellular events with one quantum dot at a time, but also to probe intracellular events (Pinaud et al., 2010). Delivering nanoparticles into the cells require to cross the cellular membrane and many protocols have been developed for this purpose (Delehanty et al., 2009). We present two strategies using endocytotic mechanisms to deliver QDots inside living cells: osmotic shock and receptor-mediated internalization.

The former internalization approach was used by Maxime Dahan's group (Courty et al., 2006; Pierobon et al., 2009). They developed a new methodology to track the motion of intracellular proteins (kinesin and myosin respectively) with a high sensitivity using semiconductor quantum dots (QDs). They incubated the kinesin or myosin-functionalized QDots complexes, which get spontaneously internalised inside vesicles and subsequently applied an osmotic shock to break the vesicles and release QDs into the cytoplasm. Kinesin or myosin-functionalized QDots are then free to move inside the cell (Hela cells) and they can be tracked using standard wide-field microscope.



Figure 2.5: Receptor-mediated internalization of QDots in endosome and its subsequent tracking in axon of DRG neurons. (a) Microfluidic platform for single fluorophore imaging of axonal transport. **(b)** Trajectories of several endosomes moving in the same axon through the same field of view. The majority of endosomes move independently (black circles). Endosomes moving together or having trajectories crossing each others are shown in red and green for clarity. The blue arrows indicate the places where some trajectories paused at the same axonal location. **(c)** Fluorescently labelled organelles that are traveling along microtubules in an axon appear as diffraction-limited spots. At each time point, the central positions of those organelles (containing a single QDots)

are determined to high accuracy by fitting with a 2D Gaussian function. As organelles travel along the axon, their time-lapse central positions trace out the microtubule tracks along which they move. Cumulative trajectories depict densely packed microtubules that are otherwise unresolvable using conventional optical imaging. From (Mudrakola et al., 2009).

Alternatively, Bianxiao Cui's group used the receptor mediated approach to deliver QDots inside neurons (Chowdary et al., 2015; Cui et al., 2007; Mudrakola et al., 2009) (Figure 2.5). They dissected retrograde axonal transport of nerve growth factor (NGF) signals by tracking quantum dot functionalized with NGF (QD-NGF) in compartmentalized culture of rat dorsal root ganglion (DRG) neurons. To perform their experiments, DRG neurons are cultured inside a microfluidic chamber (Figure 2.5a) that separates the chemical environment of the distal axons from that of the cell bodies. Fluorescent QD-NGF are applied only to the distal axons where they bind to its membrane receptor and is subsequently endocytosed and retrogradely transported toward the cell body. Pseudo-TIRF (oblique illumination) imaging setup is restricted to the microchannels, so that only internalized and transported QD-NGF molecules are observed. Using this approach, they unravelled the binary STOP&GO motion of retrograde axonal transport (Figure 2.5b) and they were able to map part of the microtubule subnetwork (Figure 2.5c).

Other inorganic probes for intracellular transport study

Semiconductor QDots are not the only inorganic probes that have been used to study of intracellular transport. Gold nanoparticles or nanorods and Single-Walled carbon NanoTube (SWNT) are other inorganic probes whose their detection relies on different physical properties. Gold nanostructures use plasmonic resonances that can be excited with a laser in the visible range with subsequent thermal increase. Gold nanorods were used to study rotational dynamics of cargos at pauses during axonal transport in PC12 cell lines (Gu et al., 2012). Practically, transferrin-coupled gold nanorods were endocytosed and the rotational dynamics were imaged using Differential Interference Contrast (DIC). Another interesting example is the use of photothermal imaging of 5 nm gold nanoparticles functionalized with a nanobody anti-GFPs for long-term tracking of proteins in COS7 cell lines (Leduc et al., 2013). The strengths of these nanobody-coated gold nanoparticles are their small size, perfect photostability, high specificity, and versatility of use against GFP-tagged proteins. However, this imaging technique requires high power laser and the internalization of this nano-complex was done by electroporation, which induced high mortality of primary neurons.

SWNT are fluorescent probes that can be excited by an infrared laser. As previously described, endocytotic methods have the disadvantage that internalized particles are entrapped in endosomal compartments, which block their ability to reach specific intracellular targets. To solve this issue, Fakhri et. al. used electroporation to internalize HaloTag-coupled near-infrared fluorescent SWNT in Cos7 cells, previously transfected with kinesin-receptor halo. This lead to the intracellular formation of SWNT-kinesin complex that can be tracked by fluorescence microscopy. With this approach, the authors were able to get a high spatio-temporal resolution mapping of intracellular fluctuations (Fakhri et al., 2014). However, as already mentioned, this experimental procedure is only applicable to cell lines which are far more robust than primary

neurons. Altogether, even though these inorganic probes brought interesting results for some biological system, to our knowledge, none of these techniques were used in primary neurons.

2.2.3 Conclusion 2.2

In this section, we detailed the standard labelling tools that are used to study intracellular transport and associated examples. From organic fluorescent proteins to inorganic probes, coupled with superresolution microscopy or optogenetic, these existing tools brought important breakthroughs in biology and neurosciences. However, none is these techniques were used to study intraneuronal transport in homeostatic native conditions. Indeed, either they overexpress some protein of interest (Bergeijk et al., 2015; Westphal et al., 2008), or they force the spatial arrangement of neurons (Mudrakola et al., 2009). Therefore, there is still a need for other alternative nanotools to quantitatively investigate the active transport inside primary neurons with high spatiotemporal resolution and without changing neuronal homeostasis.

2.3 Production, physical properties and bio-applications of fluorescent nanodiamonds

As we aim at developing a non-genetic and minimally invasive assay to spatiotemporally quantify longterm intraneuronal transport, fluorescent nanodiamonds (fNDs) seem to be a probe of choice. Indeed, fNDs are emerging as versatile probe for both *in vitro* and *in vivo* bioimaging, taking advantages of their perfectly photostable emission in the far red, their high brightness and their absence of cytotoxicity. Therefore, in this section, we first describe production and physical properties of fNDs, before reviewing recent applications of fNDs in biology. For more detailed information, readers can refer to the two following reviews on the properties and applications of nanodiamonds and their comparison with silicon quantum dots for *in vitro* and *in vivo* long-term bioimaging (Mochalin et al., 2012; Montalti et al., 2015).

2.3.1 Production of fNDs

As already mentioned, getting a stable aqueous colloidal suspension of nanoparticles is challenging and NDs are not an exception. However, fNDs have the advantages of a surface chemistry that can be modified after the last step of its fabrication by strong acid and air oxidation leading surface chemical groups (in particular carboxyl) that ensures colloidal stability in water at pH \approx 7 by electrostatic repulsion (carboxylated fNDs have a negative surface charge as evidence by a zeta-potential value of about -40 mV in water at pH=7). Furthermore, diamond displays a chemical inertness, which prevents degradation and can also be instrumental for covalent biomolecule functionalization.

Nanodiamond crystalline structure is based on tetrahedral sp³ hybridization (sp² if graphite) (Figure 2.6a). Top-down and bottom-up approaches are used to synthetize nanocrystals diamond (ND) as

detailed below. Nitrogen vacancy color centers can be created in diamond combining the presence of nitrogen atoms as natural impurities and the generation of vacancies by irradiation with high-energy particles (protons in particular). NV centres are formed after thermal annealing (see details later in this section). Finally, ND oxidative treatment allows the removal of possible graphitic shell leading to stable, water dispersible, fluorescent NDs (fNDs). Moreover, fNDs emit in the far red and near infrared region (600-800 nm), which make them ideal for low-background *in vivo* detection (Figure 2.6b). Most results also demonstrated an unprecedented high biocompatibility of fNDs both *in vitro* and *in vivo*.



Figure 2.6. Structural and emission properties of fNDs. (a) Molecular model of a single 5 nm ND after oxidative treatment purification. The diamond core is covered by a layer of surface functional groups, which stabilize the particle by terminating the dangling bonds. The majority of surface atoms are terminated with oxygen-containing groups (oxygen atoms are shown in red, nitrogen in blue; hydrocarbon chains in green and and hydrogen in white). From (Mochalin et al., 2012). (b) Comparison of the fluorescence spectrum (red curve) of NV centers in FNDs with the near-infrared (NIR) window of biological tissues. The black, dark gray, and light gray curves are the absorption spectra of H_2O , oxygen-bound hemoglobin (HbO₂), and hemoglobin (Hb), respectively. From (Vaijayanthimala et al., 2012)

Nanodiamond production: HPHT & Detonation techniques

The name "diamond" comes from the ancient greek word " $\alpha\delta\alpha\mu\alpha\varsigma$ " which mean "unbreakable". Given its unique physical properties (e.g. optical, electrical or thermal), this material allows a broad range of applications. It exists under natural and synthetic form, but for the majority of the applications, the synthetic form is used. Most NDs used for bioimaging are prepared from commercial diamond powders resulting from ball milling of diamond microcrystals produced by high pressure high-temperature (HPHT) method (Korobov et al., 2013). NDs can also be synthesized by detonation techniques (Korobov et al., 2013; Mochalin et al., 2012). Although they were not proved to be suitable for creating fluorescent NDs, other methods have been reported for ND production, such as laser ablation, plasma-assisted chemical vapor deposition (CVD), autoclave synthesis from supercritical fluids, ion irradiation of graphite, chlorination of carbides, electron irradiation of carbon onions and ultrasound cavitation (Montalti et al., 2015). HPHT technique involves large presses that produce high pressures (~6 GPa) at high temperatures (~1500°C) and yielding micrometre-size diamond crystallites (100-200 μ m). Graphite and metallic catalysts are placed in hydraulic presses for few hours, leading to graphite-diamond conversion. Then, other steps are needed to obtain nanometer-size diamonds. The most common size reduction technique consists in milling diamonds with hard balls (e.g. made of zirconium) in a planetary mill. Yet, this approach leads to irregular shape that could be responsible for breaking the membranes of endosomes (Chu et al., 2014). Finally, monodispersed diamond nano-powders with a size being hardly below 10 nm are obtained (Mochalin et al., 2012).

Another way to produce diamond nanoparticles under high-pressure high-temperature conditions is by using explosives. Indeed, smaller NDs can be prepared by detonation processes that yield aggregates of NDs with sizes of 4–5 nm embedded in a detonation soot (Danilenko, 2004; Mochalin et al., 2012). An explosive mixture (typically trinitrotoluene and hexogen in a mass ratio ranging from 40:60 to 70:30) provides both a source of carbon and energy for the conversion. The detonation chamber reaches temperature of ~3000– 4000°C and pressure of ~20–30 GPa. Additional purification steps are needed because NDs production is often impure due to contamination with other carbon allotropes, metals and oxide residuals, and generation of graphitic layers on the ND surface. What makes detonation ND unique is that surface hydrogenation of these NDs allows mono-disperse colloidal water solution with exceptional long time stability in a wide range of pH, and with high positive zeta potential (>60 mV) (Petit et al., 2013; Williams et al., 2010). However, detonationbased fNDs suffer from low efficiency of the color center formation (Smith et al., 2009) and the low photostability (Bradac et al., 2010).

Creation of NV centers in diamond

NDs do not absorb light and do not show any luminescence unless they present structural crystal defects (Aharonovich et al., 2011). Whereas the emission of QDots results from quantum confinement effects, the emission of fNDs come from lattice defects that produce localized excited states upon light absorption. Interestingly, diamonds may host over 500 kinds of photon absorbing and/or emitting defects (color centers) (Zaitsev, 2000) and some of them have been used as single-photon sources at room temperature (Aharonovich et al., 2010; Wu et al., 2006).

Diamond classification is based on its nitrogen content and its optical absorption. In general, diamonds containing nitrogen impurities are classified as type I while those with no measurable traces of nitrogen are type II (Walker, 1979). Only type I diamonds are suitable for producing intrinsic luminescent probes. In type I diamonds, the paramagnetism and optical absorption are due to the nitrogen impurities. They are subdivided into two categories, denoted type Ia (most of the natural diamonds) and type Ib (most of the synthetic diamonds). In type Ia, nitrogen is at a concentration up to 3000 ppm and clustered, whereas in type Ib diamonds, nitrogen is at a concentration is very low (<1020 atoms/cm³). It is divided into two subcategories, the type IIa and IIb. The first one is the purest diamond, colorless and free from all impurities, while the second one contains high quantities of boron atoms, leading to semi-conductor properties.

The general approach to produce bright fNDs compatible with a physiological environment and suitable for bioconjugation is schematized in Figure 2.7. This figure shows that two main strategies exist to prepare aqueous dispersions of fNDs, depending if nanometer-size reduction is done before or after the irradiation step. In both cases, the creation of NV centers in diamond crystals can be achieved by two ways: (i) with irradiation damage of type Ib diamond using high energy particle beam (electron, neutrons, ions) and subsequent thermal annealing (at temperature >800°C in vacuum); (ii) with direct implantation of nitrogen in pure (type IIa) diamonds and subsequent thermal annealing. In both cases, a surface sp² graphitic shell is formed after the thermal treatment and oxidation step is thus needed to remove it. A benefit side-effect is the creation of surface carboxylate groups which enhance the colloidal stability. It is worth underlying that fND photoluminescence is related to the nature of the defect embedded in the diamond nanocrystal which implies that the emission spectrum cannot be tuned continuously like in the case of semi-conductor QDots, by changing size.

Vacancy creation by irradiation relies on the mechanism described hereafter. As a charged particle passes through matter, part of its particle kinetic energy can be transferred by elastic collision to a lattice atom, leading to the removal of this atom from its normal lattice position. The ejected atom may cause a cascade of atomic displacements before eventually losing its kinetic energy and stop moving. This effect is significant when the incident particle is heavy (proton or alpha particle) or has an energy higher (a few MeV for electrons) than the displacement energy of the atom. For NV creation in the diamond lattice, various type of particles can be used: neutrons, alpha particles, protons or electrons. The defect creation efficiency varies with respect to the particle type and energy. Chang et al. developed a medium-energy (40 keV) He⁺ beam for mass production of NDs with high concentration of color centers. As a comparison, a single 40 keV He⁺ ion can create up to 40 vacancies as it penetrates diamonds, compared to the 0.1 and 13 vacancies generated by a 2 MeV electron and a 3 MeV proton, respectively (Chang et al., 2008).



Figure 2.7: Schematic representation of the two main strategies to prepare an aqueous dispersions of fNDs. From left to right: micro-sized diamonds (Ds) are reduced in size (NDs), then ion beam damaged and finally,

thermally treated to create color centers (Activated NDs ANDs). Thermal annealing produces a surface graphitic layer, which is eliminated by oxidation producing surface carboxylic groups together with other functions (anhydride and hydroxyl groups, not shown). In the alternative approach (from right to left), microdiamonds are first activated to produce fluorescent diamonds, which are then reduced in size and oxidized to produce fNDs. From (Montalti et al., 2015)

This irradiation step is followed by a thermal annealing step. Performed under vacuum or inert gas, this step is compulsory for NV creation and in practical situation, the sample is exposed for 2 hours at a temperature of about 800°C. At such temperature, the vacancies start migrating and stabilize themselves in the adjacent site of the nearest substitutional nitrogen atom as this position is the most thermodynamically favorable one (Iakoubovskii en Adriaenssens, 2001). In the case of nanodiamond, the optimal values of temperature and timing of the annealing process are still open questions, but one can use experiments done in bulk diamond at ENS Cachan (France) and CEMHTI (Orléans, France) laboratories as a guide (Botsoa et al., 2011). Interstitial atoms (carbon displaced during irradiation) also move upon temperature annealing and there is a competition between N-V formation and interstitials-V recombination.

For biological applications, a high density of NV centers per nanoparticle is desired, so that irradiation of type Ib diamond is preferred, while for quantum information applications, single NV center is needed, thus implantation in pure type IIa diamond is more adequate.

In all our studies, we used far-red emitting NV-center fNDs from HPHT type Ib NDs fabricated by Huan-Cheng CHANG's group at Institute of Atomic and Molecular Sciences (Academia Sinica, Taiwan) and based on the following procedure irradiation. The nanodiamonds were radiation-damaged with a high-energy (3 MeV) proton beam, as detailed in Su et al. (Su et al., 2013). A thin diamond film (thickness<50 µm) was prepared by depositing \approx 5 mg of nanodiamond powder on a silicon wafer (1x1 cm² size) and subsequently subjected to the ion irradiation at a dose of \approx 2x10¹⁶ H⁺.cm⁻². Afterwards, the radiation-damaged nanodiamonds were annealed at 800°C for 2 h to form fNDs. To remove graphitic carbon atoms on the surface, the freshly prepared fNDs were oxidized in air at 490°C for 2 h and microwave-cleaned in concentrated H₂SO₄– HNO₃ (3:1, v/v) at 100°C for 3 h.

2.3.2 Optical properties of single fluorescent nanodiamonds

The spectral region of fNDs can be tuned from the NIR to green or blue depending on the nature of the color center. **Red-NIR emitting fNDs** are broadly used in many fields of science. Here, the photoluminescence comes from the negatively charged nitrogen-vacancies (NV⁻ centers) which are produced in type Ib diamonds together with their neutral counterpart NV⁰ (Rondin et al., 2010), as shown in Figure 2.8a. These centres show distinct Zero Phonon Line (ZPL) at 637.6 nm and 575.4 nm for the negative and neutral defects respectively. ZPL is the wavelength at which an excitation/relaxation process giving PL properties is not phonon assisted. It was shown that the ratio NV⁻/NV⁰ increases with respect to the size of the NDs and is enhanced when graphitic defects on the surface are removed. Another type of fNDs is the **green emitting fNDs**, which is produced in type Ia diamonds where the N atoms are clustered in the structure. Here the complex N-V-N

shows a ZPL at about 504 nm and a maximum absorption in the blue at 470 nm. This center emits exceptionally stable green fluorescence with a maximum center on 531 nm with a fluorescent quantum yield close to 1, an excited state lifetime of 16 ns and no photoblinking (Hsu et al., 2011; Wee et al., 2009). Eventually, the **blue emitting fNDs** stem from the N3 centers, which are composed of three nitrogen atoms surrounding a vacancy (Davies et al., 1992). The formation of N3 color center uses the same procedure as for creating NV center but with a pre-treatment with He⁺ ions followed by molecular N₂⁺ ions beam (Himics et al., 2014). Finally, it is worth noting that other types of defects color centers can be found in fNDs. For example, embedded **silicon-vacancy** (Si-V) color centers were created in NDs, grown by microwave plasma-enhanced chemical vapor deposition (CVD) (Neu et al., 2011). Negatively charged SiV⁻ centers have a strong narrow-band fluorescence at room temperature with a 738 nm ZPL. SiV color centers in NDs have been recently observed by cathodoluminescence (Zhang et al., 2014). One basic example of bioapplication of SiV was shown in (Merson et al., 2013).



Figure 2.8: Photophysical properties of NV-color center fNDs. (a) Normalized PL spectra of single NV- and NV^o color centers in NDs. The zero-phonon line (*) is an intrinsic feature of NV- (637 nm) and NV^o (575 nm). The inset shows the atomic structure of the NV defect, consisting of a substitutional nitrogen atom N associated with a vacancy V in an adjacent lattice site of the diamond crystalline matrix. From (Rondin et al., 2010). (b) Resolving multiple adjacent defects located in single NDs using STED super-resolution microscopy. STED rasterscan (blue to red colors) is superimposed to scanning electron microscopy image (grey color) of the same nanocrystal. From (Arroyo-Camejo et al., 2013). (c) Photostability of fND (red) versus fluorescent polystyrene nanospheres (blue) excited under the same conditions. From (Yu et al., 2005).

From now on, we will only focus on the most famous fNDs, namely Red-NIR emitting ones. As shown in Figure 2.9a with a schematic description of the energy levels of the NV- center, the ground and excited states are triplet spin states of multiplicity type ³A and ³E respectively. There is also a metastable singlet state ¹M of intermediate energy. When a photon is absorbed by the NV- center, the system is excited to the ³E state. From this state, either the system falls back to the ground state, or is trapped in a metastable state ¹M if an intersystem crossing transition occurs. The decay rate ³E \rightarrow ¹M (0.005 ns⁻¹) is low in comparison to the decay rate of the ³E \rightarrow ³A transition (\approx 0.03 ns⁻¹) so de-excitation mostly occurs through the ³E \rightarrow ³A photoluminescent transition. Thanks to their perfect photostability (Figure 2.8c), NV defects can be investigated at the single emitter level and STED microscopy can be pushed to its record of resolution. Doing so, Stefan Hell's group succeeded to resolve NV center down to 2.4 nm (Wildanger et al., 2012), and ENS Cachan team, in collaboration with Hell's team later showed that multiple adjacent defects located in single NDs can be imaged individually (Figure 2.8b) (Arroyo-Camejo et al., 2013).

The brightness of individual fNDs depends on three main features: the fluorescence quantum yield, the molar absorption coefficient and the number of color centers per ND. NV-color center fNDs quantum yield η and fluorescence lifetime τ have been inferred by the team of Femius KOENDERINCK and showed a broad dispersion of η from 10% to 90% and τ from 20 ns to 40 ns, η being partly affected by unfavourable orientation of NV dipole relative to the surface ND (Mohtashami en Koenderink, 2013). The molar absorption coefficient of fNDs has been inferred by the team of Taras PLAKHOTNIK with an absorption cross-section of about 0.95±0.25 x 10⁻¹⁶ cm² (Chapman en Plakhotnik, 2011) which is of the order of magnitude of organic dyes (Table 2.1). The number of color centers produced in each ND is dependent on the fabrication process, but the largest concentration achieved to date in the team of H.-C. CHANG in Taiwan, correspond to about 15 NV centers per fND of size≈30 nm and 500 in fND of size ≈100 nm as estimated from overall fluorescence intensity comparison to single emitter reference.

A direct comparison of the photoluminescence properties of a single QDots and fNDs with a single NVcolor center demonstrated that, at saturation of their excitation, PL intensity of fND with a single NV-color center is only three times smaller than the one of a single QDots (Faklaris et al., 2009a). However, the excitation power to reach the saturation level was two order of magnitude higher for fND than QDots.

Table 2.1 summarizes the fluorescent properties of organic probes, QDots and fNDs and other characteristics such as the physical properties (e.g. size, fluorescence stability...), production processes or biological properties (e.g. toxicity...).

Property	Typical organic Dyes	QDots	fNDs
Size	<1nm	3–10 nm	>4 nm
Emission spectrum	IR-UV, selected by length	IR-UV, selected by size	IR-UV, selected by the defects
Emission line width (FWHM)	35–100 nm	30 – 90 nm	mostly >100 nm
Absorption Cross Section	Typically 1×10 ⁻¹⁶ cm ²	Typically 3×10 ⁻¹⁵ cm ²	Typically 1×10 ⁻¹⁶ cm² (a)
Quantum Yield	0.5–1.0	0.1–0.8	0.1–0.9 (b)
Lifetime	1–10 ns	10–100 ns	25 ns
Photostability	Low	High	Extremely High
Thermal Stability	Low	High	Extremely High
Toxicity	medium to high	High	Low
Synthetic Processes	Easy	Mostly Hard	Very Hard
Commercialization	Yes	Yes	Yes (c)

Table 2.1: Comparison of the fluorescent properties of organic dyes, QDots and fNDs. Values are from (Montalti et al., 2015; Schirhagl et al., 2013), except for (a) from (Chapman en Plakhotnik, 2011); (b) from (Mohtashami en Koenderink, 2013); (c) sell by Adamas and Sigma-Aldrich.

Apart from the PL property of fNDs, the unique quantum properties of the NV spin can also be instrumental for *in vitro* and *in vivo* imaging (readers can refer to the following review (Montalti et al., 2015; Schirhagl et al., 2013)). Indeed, NV-center fNDs presents degenerated spin energy states where the triplet state is split into two energy levels with $m_s = 0$ and $m_s = \pm 1$. The energy diagram of NV center in Figure 9a shows the fine structures of both fundamental and excited states, as well as the metastable state. Here, the continuous (resp. dashed) arrows point out radiative (resp. non-radiative) transitions. Owing to strong non-radiative transition from $m_s = \pm 1$ toward the metastable state and then the ground state, $m_s = \pm 1$ is defined as a dark state whereas $m_s = 0$ is the bright state. As a consequence, two different ways to emit fluorescence are possible, with a quantum yield which differ between each other of about 20% (Epstein et al., 2005) (Figure 2.9b). Moreover, the spin state can be tuned with a radiofrequency (rf) wave and directly monitored through a PL variation (Figure 2.9b). In addition, a variation of the surrounding magnetic field or temperature will change the population configuration of the two sub-level, which can also be monitored through PL variations. An example of application is shown in Figure 2.9c in which spin resonance spectra of a single NV center is acquired under different external magnetic field, which creates a Zeeman effect. This last phenomenon is performed in the magnetometer (Le Sage et al., 2013).



Figure 2.9: Optically detected magnetic resonance. (a) Energy diagram of NV center showing fine structures of both fundamental and excited states, as well as the metastable state. Continuous (resp. dashed) arrows point out radiative (resp non-radiative) transitions. **(b)** The spin state can be tuned with a radiofrequency (rf) wave (top) and directly monitored through a PL variation as $m_s = 0$ is a bright state whereas $m_s = \pm 1$ is a dark state (bottom). **(c)** Spin resonance spectra of a single NV center showing variation of the PL with respect to the rf frequency for different external magnetic field, due to Zeeman effect. This phenomenon is exploited for nanomagnetism recording. Adapted from (McGuinness et al., 2013; Tetienne, 2014).

2.3.3 Biological applications of fNDs

fNDs fluorescent probe combines photostability and biocompatibility which make it possible to achieve extraordinary broad range of applications in various scientific fields (Schirhagl et al., 2013). In this subsection, we mainly detail both *in vitro* and *in vivo* biological applications. However, theranostic applications of NDs and fNDs is not discussed here (readers can refer to the following reviews (Montalti et al., 2015; Perevedentseva et al., 2013a). Eventually, we conclude this subsection with a brief detour on its use as a nanosensor in biology via the manipulation of the NV spin, which makes this probe unique.

fNDs biological applications using fluorescence properties

fNDs production process leads to carboxylated surface (after the cleaning stage), which is in the negatively charged form -COO⁻ at pH=7. When mixed with the cell culture medium supplemented with serum, fND are quickly covered by a protein corona (Tenzer et al., 2013). fNDs were first used in non-targeted *in vitro* cellular imaging applications (Fu et al., 2007; Neugart et al., 2007; Yu et al., 2005), (Wee et al., 2009). Thereafter, *in vivo* imaging of bare fNDs was also achieved in C-*elegans* using standard epifluorescence microscopy (Mohan et al., 2010) (Figure 2.10a). However, because of light scattering from the tissue, standard microscopy techniques show limitations. Therefore, other fluorescence features of NV⁻ centres in nanodiamond, such as long fluorescence lifetime (>25 ns) or sensitivity to magnetic field, can be instrumental to increase the fND imaging contrast. Therefore, FLIM (Fluorescence Life Time Imaging) was used to study intercellular transport of yolk lipoproteins in C-*elegans* (Figure 2.10b) (Kuo et al., 2013). FLIM imaging of fNDs was also used to track lung stem cells *in vivo* in a mouse model and helped to understand how they incorporate and contribute to tissue regeneration (Hsu et al., 2014; Wu et al., 2013) (Figure 2.10c).

Let us finally mention cathodoluminescence as another imaging modality of fNDs, in which colour centres in diamond luminescence are excited with a focused electron beam, serving at the same time to image nanoparticles and their environment with the nanometer resolution of electron microscopy. Moreover, all colour centres can be excited with the same electron beam allowing multicolor imaging. This approach was applied in proof-of-principle experiments to integrated and correlated imaging of fND in cells together with their ultrastructure (Glenn et al., 2012; Nawa et al., 2014; Zhang et al., 2014).



Figure 2.10: Biological applications of fNDs using their fluorescence properties. (a) C-elegans fed with dextran-coated fNDs (red). (Inset) A 100×magnified image showing fNDs within the intestinal cells. From (Mohan et al., 2010). (b) Observation of GFP::YLC and fNDs in *C.* elegans by FLIM. From (Kuo et al., 2013). (c) Identification of transplanted fND-labelled Lung Stem Cells in bronchiolar epithelia using FLIM. From (Wu et al., 2013).

fNDs biological applications using NV-center nanoscale sensors

The aforementioned ODMR is another unique property of NV- centre fNDs. (Figure 2.10). This quantum feature was applied to reduce the tissue autofluorescence background to perform *in vivo* imaging. By ON-OFF modulation of the microwave field, it is possible to detect only the fNDs, as demonstrated in C-*elegans* and rat (Igarashi et al., 2012). By applying a gradient of magnetic field, like in Magnetic Resonance Imaging, Hegyi and Yablonovitch showed that fNDs can be imaged through scattering and autofluorescent tissues (Hegyi en Yablonovitch, 2013). Eventually, a simple magnetic modulation also allowed to enhance the signal-to-background ratio up to 100-fold in *in vivo* imaging (Sarkar et al., 2014). Although modulation of the NV center emission using microwave and magnetic field still needs technical improvements to apply them to relevant biological questions, non-optical modulation of fND emission is a very promising approach to perform background-free *in vivo* imaging.

Other applications of the unique quantum property of fNDs was performed in Hela cells for quantum measurement and orientation tracking *in vitro* (McGuinness et al., 2011). Then, the first biological application of fNDs-based magnetometry was performed in bacteria (Le Sage et al., 2013; Steinert et al., 2013) and this approach was applied in genetic for probing the functional diversity of magnetosome formation (Rahn-Lee et al., 2015). Moreover, its thermometer feature was demonstrated in human embryonic fibroblast where fNDs were deliver jointly with gold nanoparticles as local heaters (Kucsko et al., 2013). Eventually, numerical

simulations seem to prove the benefit of measuring the quantum decoherence of NV-center fNDs for the monitoring of ion-channel function in real time (Hall et al., 2010; Kaufmann et al., 2013) as well as for high spatiotemporal resolution wide-field imaging of neuron activity (Hall et al., 2012). However, no proof-of-principle were performed so far in real neurons.

2.3.4 Conclusion 2.3

In this section, we described in detail the production, physical characteristics and biological application of NV-center fluorescent nanodiamonds. From fluorescent properties to quantum features, fNDs were shown to be an overwhelming inorganic fluorescent probe for bioimaging with a lot of advantages compared to standard nano-source of light. Chemical inertness, emission in the NIR region and biocompatibility make it possible to detect fluorescence from fNDs in the long term for *in vitro* and *in vivo* imaging. Overall, due to its photophysical properties such as a perfect photostability and a high brightness, it seems that fNDs is the probe of choice to non-genetically monitor the intraneuronal transport for a long period of time with high spatiotemporal resolution. However, fNDs were never used for quantitative studies at the molecular level and no approaches were conducted in living neurons dissociated from brain of mouse embryo? Is fND toxic for primary neurons? Which optical system can be used to address this question?

2.4 Live-cell imaging for fNDs tracking inside primary neurons

In this section, we describe the experimental approach we used to monitor intraneuronal transport. We first discuss about the neurobiological and optical tools suitable for our study, namely primary culture of neurons and the live-cell imaging setup. Then, we explain how we can efficiently deliver fNDs inside hippocampal neurons. Uptake mechanism and cytotoxicity of fNDs are discussed.

2.4.1 Primary neuron culture from mouse embryo

As we aim at evaluating the functional impact on intraneuronal transport of psychiatric risk factor, neurons have to be used as the substrate of our study. Indeed, cell lines contain a mutant genome with the presence of gene recombination and huge number of gene copies. Moreover, even cell lines derived from central nervous system precursors have limitations because the neurons derived from these lines cannot form well-defined axons, dendrites and synapses, all mandatory to establish a functional network of neurons. One of the best animal model available to study psychiatric disorders is the mouse as it is genetically closer to human than *C-elegans*, drosophila or zebrafish, mainly used for *in vivo* study of connectomics and embryogenesis.

The hippocampus and cortex are complex structures, which make them challenging to analyse and manipulate *in vivo*. Instead, primary cell culture techniques have been successfully developed to study these

neurons *in vitro*. One of the most well-established and widely used techniques for the study of hippocampal and cortical neurons is the primary neuron culture from mouse embryo, whose main steps are shown in Figure 2.11 (Banker en Cowan, 1977; Kaech en Banker, 2006). This culture system allows neurons to be cultured *in vitro* in a far less complex environment than that used *in vivo* (i.e. without glial cells and blood vessel for example), making them highly accessible for further manipulations and observations. The vast majority of cells in these preparations are excitatory pyramidal neurons, with inhibitory neurons representing about 18–20% of the population. The dissociated embryonic neurons maintained in culture undergo distinct stages of differentiation and form well-established synaptic connections (Beaudoin et al., 2012). These cells can be grown and maintained in single layers on glass coverslips and subsequently observed by fluorescence microscopy imaging. Gene expression can also be manipulated to dissect plethora of neurobiological mechanisms (see Chapter 4, Section 4.2).

Some laboratories perform primary neuron culture on an astrocyte feeder layer. This allows for the neurons not to be in direct contact with the astrocytes, but to be exposed to growth factors they have secreted into the medium. In our lab, we use an alternate protocol that does not involve the use of the feeder layer. Instead, the culture medium is complemented with serum-free medium (B27) supplemented with cofactors necessary for neuronal growth and maintenance (Brewer et al., 1993).



Figure 2.11: Primary culture of hippocampal neurons. Left: main steps of the technique from brain extraction of the mouse embryo, to the dissection of hippocampus and neuron dissociation using chemical compound. Right: Phase-contrast images of hippocampal cultures after 1 day and 13 days in culture showing different developmental stage. Non-neuronal cells can be plated with neuronal cells (arrow). Scale bar, 50 μ m. (Beaudoin et al., 2012)

In vitro development of neurons has well-defined developmental stage with axon/dendrite polarization after DIV 7, then immature dendritic spines around DIV 12, synaptic connection after DIV 15 and well established neuronal network around DIV 21. In the rest of the study, we will focus only on hippocampal neurons, which give a homogeneous pyramidal neuron culture. A fully detailed protocol of primary culture of hippocampal neurons dissociated from mouse embryo is presented in Appendices.

2.4.2 Pseudo-TIRF microscopy for live cell imaging

Physicists have developed for a long time powerful microscopes to get biological samples under scrutiny. Invented in 1665 by Robert Hooke, an English physicist, architect and natural philosopher, the microscope has undergone tremendous innovation and revolution. The last breakthrough in the 2000's was achieved by the development of super-resolution microscopy, which was rewarded in 2014 by the Nobel Prize in Chemistry. When the field of microscopy meets the field of neurosciences, we obtain overwhelming discoveries, whose the first one was achieved by the Spanish neurobiologist Ramon y Cajal in 1890's with the discovery of neurons and rewarded in 1906 by the Nobel Prize in Physiology or Medicine. The microscope becomes even more compulsory when dealing with Neurosciences because neurons are one of the most complex cell types in the human body with the presence of submicron functional compartments, namely post-synaptic and pre-synaptic part of a synapse.

One important issue when imaging a fluorescent dye is to choose the right microscopy technique, as a trade-off have to be made between spatial and temporal resolution of imaging. One of the easiest microscopy technique to implement, after Epifluorescence microscopy, is the Total-Internal Reflection Fluorescence (TIRF) microscopy (Axelrod, 1981). This technique is based on the Snell-Descartes law, which states that at a specific angle (called the critical angle θ_c) to the interface between two media with different refractive index, the entrance beam undergoes a total reflection with the same angle. In another word, no transmitted light will be detected at this angle. However, at the contact point of the interface, if considering a glass coverslip and water as being the two interface, an evanescent wave will propagate into the water for a distance *d* which follow the mathematic expression below:

$$d = \frac{\lambda}{4\pi * \sqrt{n_{glass}^2 \cdot \sin(\theta)^2 - n_{water}^2}}$$
(2.1)

where *d* is the penetration depth and is dependent upon the wavelength λ of the incident beam, the angle of incidence θ , and the refractive indices *n* of the two medium n_{glass} and n_{water} .

Practically, a wide range of optical arrangements for TIRF microscopy can be used, such as prism or high NA objective configuration. Here, we will only detail the "prismless" configuration to understand how a TIR effect is obtained. The incident beam must be constrained to pass through the periphery of the objective's pupil and must emerge with only a narrow spread of angles. This can be accomplished by setting the incident beam to be focused off-axis at the objective's back focal plane (Figure 2.12). The farther from the off-axis, the larger the output angle.

On the one hand, the condition to not have any refracting beam is that $\theta > \theta_c$, for which Snell-Descartes law becomes:

$$n_{\text{water}} = n_{\text{glass}} \cdot \sin(\theta_c)$$
 (2.2)

On the other hand, the incident beam will emerge from the immersion oil of refractive index n_{oil} at a maximum possible angle θ_m (measured from the optical axis) given by:

$$NA = n_{\text{oil}} \sin(\theta_m)$$
 (2.3)

From equations 2.2 and 2.3, and by matching the oil refractive index with the glass one, we perfectly understand why having objective NA greater than n_{water} with a substantial margin is preferable. The highest aperture available is an Olympus 100X NA = 1.65, which allow higher flexibility and penetration depth. However, the mismatch of refractive index will produce spherical aberrations, and thus it is mandatory to use coverslips and objective oil with higher refractive index, which both have drawbacks. As a good compromise, we will use Nikon 100X NA = 1.49 and standard coverslips and oil with n = 1.518. For TIRF alignment, once the beam is aligned to the optical axis and focused at the back focal plan of the objective (epifluorescence configuration), the illumination angle is continuously switched all the way from standard epi to TIR simply by increasing the off-axis position of the beam (Figure 2.12). Once TIR is achieved, further increase in off-axis position will increase the incidence angle and thereby decrease the depth of the evanescent field (equation 2.1). In our experimental conditions, $n_{\text{glass}} = 1.518$ and $n_{\text{water}} = 1.33$ which lead to $\theta_c \approx 62^\circ$ (equation 2.2), and the maximal angle achievable with our objective of NA=1.49 is $\theta_{\text{max}} \approx 79^{\circ}$ (equation 2.3). Therefore, given that λ_{exc} = 561 nm, the penetration depth at $\theta_c \approx 62^\circ$ is about 269 nm whereas at $\theta_{\text{max}} \approx 79^\circ$, the penetration depth is only about 66 nm (equation 2.1). Therefore, as we want to monitor the intraneuronal transport and that neurites can be as big as few micrometre in diameter, TIRF configuration does not seem to be a relevant approach.

Therefore, we decided to use **oblique illumination** which is a technique related to TIRF that is often used in SPT and localization microscopy (Figure 2.12). The angle of the incident excitation laser beam was kept to be slightly less than the critical angle $\theta_c \approx 62^\circ$ required for complete TIR. While most of the light was reflected at the glass-water interface like in TIRF illumination, the pseudo-TIRF also resulted in a highly refracted beam that propagates at a very small angle from the glass-water surface. This pseudo-TIRF configuration offers the advantage of high signal-to-background ratio of traditional TIRF, while at the same time penetrates deeper into the sample.

We implemented the oblique illumination on a two-stages inverted microscope (Eclipse Ti-E, Nikon, Japan), equipped with 100x magnification and NA 1.49 immersion oil objective (CFI Apo TIRF 100X Oil, Nikon, Japan). The excitation beam from a continuous-wave diode-pumped solid-state laser emitting at the wavelength of 561 nm with a maximum power of 100 mW (SLIM-561-100, Oxxius S.A., France) was expanded

16x. The maximum laser intensity was 1 kW/cm² in the sample plane. We used filters adapted to the spectral characteristics of NV color centers in diamond: a dichroic beamsplitter with a steep edge at 561 nm and a 97% flat transmission above this wavelength (zt561rdc, Chroma Corp., USA) and a band-pass filter centered on 697 nm wavelength with a full-width at half maximum (FWHM) of 75 nm (HC 697/75, Semrock, USA), on the detection channel. White-light illumination observations were done with the differential interference contrast mode (DIC) of the microscope without changing the microscope objective. Images and videos were recorded with a cooled Electron Multiplied CCD array detector (iXon-DU885, Andor Technology, Ireland). We calibrated the EMCCD pixel size at 100x magnification for which one pixel corresponds to a length of 80 nm in the sample plane. Eventually, because it is compulsory to maintain a temperature of 37°C, 5% partial CO₂ pressure and 100% hygrometry to perform live neuron imaging, the whole microscope was enclosed in a cage incubator (Okolab, Italy). All the dynamic intraneuronal transport studies that are shown in this thesis were acquired using this setup.



Figure 2.12: Scheme of the live cell imaging set-up. L: *lens; BE: beam expander; DM: dichroic mirror; BFP: back focal plane. The microscope stage is enclosed inside an incubator to perform live cell imaging (37°, 5% CO₂).*

2.4.3 fNDs delivery inside neurites of hippocampal neurons

Incubation of cells with nanoparticles in the external medium often leads to their internalization by nonspecific endocytosis, depending on factors such as the size, shape, surface chemistry, concentration, cell type and the duration of incubation (Albanese et al., 2012; Kim et al., 2013). In this subsection, we describe

the strategies we used to deliver fNDs inside neurons. Both internalization pathways and cytotoxicity of fNDs *in vitro* and *in vivo* are discussed.

Internalization of fNDs and uptake mechanisms

Many routes lead to intracellular compartment and many parameters can affect the uptake efficiency of nanoparticles (Figure 2.13). Among others, nanoparticle size, shape or surface chemistry may affect the delivery of nanoparticles as well as the degree of their accumulation in the cellular compartments and their cytotoxicity (Canton en Battaglia, 2012; Montalti et al., 2015). Various experimental studies of nanoparticle internalization pathways have been conducted, but, we will only focus here on the ones which dissect fNDs delivery pathways (Chu et al., 2014; Faklaris et al., 2009b; Weng et al., 2012).



Figure 2.13: Cellular uptake pathways for nanoparticles. (a) phagocytosis, (b) macropinocytosis, (c) clathrin-mediated endocytosis, (d) caveolin-mediated endocytosis, (e) non-clathrin- and non-caveolin-mediated endocytosis. (f) Parameters that regulate cellular uptake. From (Montalti et al., 2015)

Kinetics of the uptake of transferrin or amino group functionalized fNDs by HeLa cells demonstrated that the receptor-mediated endocytosis was more effective than the endocytic process involving surface electrostatic interactions (Weng et al., 2012). The internalization pathways of 25 nm fNDs in HeLa cancer cells were also investigated by selectively blocking the different possible internalization pathways with specific

drugs. These experiments, conducted in the TREUSSART's group, demonstrated that fNDs enter HeLa cells mainly by clathrin-mediated endocytosis and that some of the smallest particles appeared to be free in the cytosol (Faklaris et al., 2008, 2009b). These results were confirmed elsewhere with cancer and non-cancer cell lines and 100 nm fNDs (Perevedentseva et al., 2013b).

Strategies to internalised fNDs inside hippocampal neurons

All studies of cellular uptake of fNDs were conducted in cell lines such as Hela cells and not in primary neuron cultures. Even though neurons are quite different from cell lines, we can assume that the internalization process described in Figure 2.12 will occur in a similar way. Therefore, we first decided to make use of this endocytosis process occurring in primary neurons (Blanpied et al., 2002). Two of the three approaches tested are described in Figure 2.14a.

The **first strategy**, proposed by Dr. LEPAGNOL-BESTEL, consists in incubating neurons with fNDs in 15 mL falcon for 30 min at 37°C, right after their mechanical dissociation from mouse embryo but before plating them onto the coverslip. Initially, the excess of fNDs was not washed out to avoid any cellular damage, but non-internalized fNDs were responsible for background fluorescence. Therefore, in a second approach, we decided to apply a centrifugation washing step. As shown in Figure 2.14b,c, the centrifugation step eliminates the excess of extracellular fNDs (white arrow), whereas internalized fNDs are more numerous (yellow arrows). The internalization pathway was investigated by studying the level of colocalization between TGN38 (Girotti en Banting, 1996) and fNDs using a confocal microscope. This experiment was performed by Dr. Marie-Pierre ADAM and readers can refer to Chapter 3 Section 3.2 of her PhD dissertation for more details (Adam, 2013). The conclusion was quite disappointing as less than 10% of internalized fNDs did colocalized with TGN38. Unfortunately, even though fNDs were successfully internalized inside primary neurons, this strategy does not give any intraneuronal transport of fNDs. This might be because fNDs are internalized too early and after few days, they got parked somewhere inside the cell or even exocytosed.

Therefore, the **second strategy** we developed in the lab consists in incubating fNDs few days after having plated the neurons (Figure 2.14a). This internalization approach has the advantage to be performed at various day in vitro (DIV). We succeeded in delivering fNDs very efficiently into neurons from DIV8 to DIV20 days in culture. To carry out the intraneuronal transport experiments, we chose to deliver fNDs between 14 and 17 days in culture because dendritic spines and neuronal network are more functional after 13/14 days (Kaech en Banker, 2006) and endocytotic function is less efficient in older neurons due to altered clathrin dynamics after 17/18 days (Blanpied et al., 2003). The precise internalization protocol we used for all the results that will be presented in Chapter 3 and Chapter 4 is detailed in Appendices.



Figure 2.14: Internalization strategies based on endocytosis mechanism. (a) Two distinct strategies of fNDs internalization inside hippocampal neurons. **(b)** DIV1 and **(c)** DIV3 hippocampal neurons with 35 nm fNDs internalised in the soma. Images are superimposed with white-light image and Alexa488-phalloïdine image respectively. Scale bars: 5µm.

A **third strategy** we tried to deliver fNDs inside the cell was based on the whole-cell patch-clamp approach (collaboration with Dr. Brigitte POTIER, at the Centre de Psychiatrie et Neurosciences, INSERM UMR 894, Paris) (Figure 2.15). Different from microinjection, this approach allows electrical activity recording along with internalization of fNDs through the patch pipette. This strategy was tested because we were supposed to get fNDs covalently functionalized with anti-GFP nanobody within my first year of PhD (see Figure 5.1). The idea was to specifically target and track FMRP-GFP proteins using this nanotool. Indeed, this protein is required for RNA granule active transport and has been shown to be impaired in Fragile X Syndrome (Alpatov et al., 2014; Brown et al., 2001). This strategy has the advantage to escape the endosomal pathway and preliminary results obtained were encouraging. However, we decided to focus the rest of our study on the spontaneous endosomal internalization, as covalently functionalized fNDs were difficult to produce and characterize.

A preliminary experiment was conducted on brain slices of rat. Patch pipette was filled with Rhodamine-Dextran and 25 nm fNDs, the former helping to re-localize the patched neuron for microscopy acquisition. Hippocampal neuron from the hippocampus CA1 region was patched for 90 min, a time long enough to be sure fNDs diffuse throughout the patched membrane. The 300 µm-thick brain slice was fixed in PFA 4%/Sucrose 3% overnight at 4°C and additional slicing of 50 µm thickness was done right after. Confocal imaging of Rhodamine (Figure 2.15b left) and Epifluorescence imaging of fNDs (Figure 2.15b middle, right) were performed to evaluate the internalization efficiency of fNDs. Even though Rhodamine photobleached, the perfect photostability of the red signal reveals the presence of fNDs. Unfortunately, it seems that fNDs were mostly aggregated in the soma, where the pipette was placed.



Figure 2.15: Whole-cell Patch Clamp strategy for fNDs delivery. (a) Top: electrophysiology set-up with a CMOS camera (top), the micropipette holder (left) and the neuron culture at DIV21 (yellow rectangle). Bottom: camera field of view during neuron electrophysiology recording. (b) fNDs delivery in one hippocampal neuron in a brain slice of the CA1 region (dashed white line). Pipette was filled with both Rhodamine (left) to re-localise the patched neuron and 25 nm fNDs (middle). Zoom of the dashed white circle region is shown in right, with white arrows pointing out neurons and the dashed white circle and triangle to help recognize the nucleus and the soma respectively. Scale bar from left to right: 50 µm, 25 µm and 5 µm. (c) fNDs delivery in one hippocampal neuron of primary neuron cultures at DIV21 as observed by Leica Confocal microscopy. Pipette was filled with both Alexa488 dye (left) and 100 nm fNDs (right). The internalization did not work. Scale bar: 5 µm.

Therefore, we decided to test this approach directly on mature primary neuron cultures (at DIV21) as it will be easier to image the cell (Figure 2.14c). A culture with well-established network is mandatory as we analyzed electrical signals to understand the state of the membrane opening and the health of the patched neuron. The pipette was filled with Alexa488-hydrazide (LifeTechnology, A-10436) and size≈100 nm fNDs. Neurons were patched for 30min and fixed in PFA 4% for 20 min at room temperature. Unfortunately, as shown in Figure 2.15b-c, no fNDs seem to be delivered inside neurons. However, because too few experiments were conducted to unambiguously observed fNDs inside neurons, further optimization are needed. This negative result might be due to fNDs aggregation inside the pipette or more basically because the commercial Leica Confocal setup in the lab is not well adapted to detect size≈100 nm fNDs (dwell time for the raster scan is not slow enough to accumulate photon and power laser in not high enough). Therefore, as covalently functionalized fNDs were difficult to product, we decided to focus our study on spontaneous endosomal internalization.

Altogether, the best internalization strategy, which allows reproducibility and high throughput, is the second strategy which consists in incubating fNDs directly in the culture medium at variable neuron

development stages. Therefore, starting from this point, we will only consider this strategy (unless otherwise stated).

In vitro and in vivo cytotoxicity of fNDs. Application to neurons

Cytotoxicity of fNDs was assessed since the first biological application by H.-C. CHANG's group in 2007 (Fu et al., 2007), demonstrating the short-term low toxicity of fNDs. Then, cytotoxicity of fNDs was investigated in more details by considering parameters such as the incubation time, nature of the cells, method of production and functionalization of fNDs, as well as their shape and size (Schrand et al., 2007; Zhu et al., 2012). Moreover, fNDs were shown not to produce reactive oxygen species, a chemophysical mechanism at the origin of the cytotoxicity of some organic fluorophores.

Usual assays to study *in vitro* cellular cytotoxicity are based on the monitoring of mitochondrial function (MTT) or luminescent ATP production with respect to various input parameters (e.g. time after incubation, concentration of fNDs...). Also, other readouts can be used such as neurite length, number of living cells for *in vitro* study and specific stress biological markers for *in vivo* study (Mohan et al., 2010).

Other studies also reported no cytotoxicity effect of bare fNDs mostly in Hela cells (Faklaris et al., 2008; Vaijayanthimala et al., 2009). However, still on Hela cells, when fNDs are functionalized with either transferrin or amino groups, the inhibition of the cellular growth (about 50% after 24 h) was observed, except for bare fNDs with carboxylic acid group on its surface (Weng et al., 2012).

We also studied the cytotoxicity of fNDs on primary culture of mouse embryonic neurons(Figure 2.15a). Here, we qualitatively studied the effect on neuronal morphology of both 30 nm and 100 nm at a concentration of 25 μ g/ml, using the first strategy. This strategy is suitable here because the centrifugation step helps to discriminate neurons with fNDs inside from those without. This classification is crucial as not 100% of freshly dissociated neurons are internalized by fNDs, leading to potential biases of blind study. In our case, we did observed morphological impairment with size \approx 100 nm fNDs but not with size \approx 30 nm fNDs. Interestingly, during the course of this work, a paper was published dealing with the effect of 100 nm-size fNDs on neuronal survival and morphogenesis (Huang et al., 2014). Almost no neuronal toxicity were reported except for concentration larger than 25 μ g/ml, which is consistent with our preliminary qualitative results.



Figure 2.16: Qualitative and quantitative cytotoxicity assessment in primary neurons isolated from mouse embryo. (a) Qualitative analysis performed in the lab (fND in red; actin in green). Top: internalization of size \approx 100 nm fNDs (white arrow head) reduce neurite outgrowth compare to fNDs-free neurons where longer neurites and growth cones lamelipodia can be observed (white dots). Scale bar: 5 µm. Bottom: internalization of size \approx 30 nm fNDs (white arrow head) does not seem to reduce neurite outgrowth and growth cones lamelipodia. Scale bar: 5 µm. (b) Quantitative analysis reported in (Huang et al., 2014). 100 nm-size fNDs reduced neurite outgrowth in a dosage-dependent manner in dissociated hippocampal neurons. Top: merged images of dissociated hippocampal neurons treated with 25 µg/ml (left) and 250 µg/ml (right) of fNDs 4 hours after seeding and incubated for 3 days in vitro (fND (red) and b-III-tubulin staining (green)). Scale bars represent 100 µm. Bottom: quantification of total neurite length per neuron in fND treated hippocampal neurons (* p = 0.05, ** p = 0.01, *** p = 0.001). Bar graph is expressed as mean +/- SEM

Observation of a STOP&GO binary motion inside neurites

As already mentioned, primary neurons are particularly difficult to study because of the low efficiency with which exogenous compounds, such as expression vectors and nanoparticles, are internalized. However, as shown in Figure 2.17, application of strategy #2 is very efficient to deliver fNDs inside freely developing branches of hippocampal neurons dissociated from brain of mouse embryos. The **experimental pipeline** on which we converged is shown in figure 2.17a. It was possible to study the movement of mean size=30 nm NV-center fNDs entrapped in endosomes of living hippocampal neurons using pseudo-TIRF microscopy in a high-throughput manner. Indeed, around 40 trajectories are effectively taken into account for final statistical tests, given that a lot of trajectories are filtered out before this point (e.g. trajectory too short, too dim, too much split...) (see Chapter 3). Usually, image acquisition of fNDs transport is done by focusing on the distal portions of neuronal branches displaying low mesh density as displayed in Figure 17b. The latter displays white-light illumination image of a main dendrite and its smaller branches, merged with one movie frame of fNDs motion,

pointing out four fNDs (yellow arrows) which move retrogradely. Snapshots are depicted on Figure 17b (right) showing two fND-labeled endosomes moving in the same dendrite (from the white rectangle). Figure 2.17c shows the kymograph for the motion of these fNDs, highlighting the "stop-and-go" characteristics of organelle transport in neurons (Overly et al., 1996). The "go" phase corresponds to directed motion mediated by molecular motors, whereas the "stop" phase may involve different mechanisms ("tug-of-war" situation of motors driving the endosome (Hancock, 2014), motor detachment from the microtubule or steric hindrance on the fND-containing endosome). This show for the first time proof-of-principle for a simple methodology to deliver fNDs inside neurites of living hippocampal neurons to non-genetically study intraneuronal transport.



Figure 2.17: STOP&GO movement of fNDs-containing endosomes inside branches of living hippocampal neurons. (a) Experimental pipeline from hippocampal neuron culture to fNDs-containing endosome trajectory acquisition using pseudo-TIRF microscopy. (b) Left: white-light illumination image of the dendritic branches merged with the fluorescence channel showing four fNDs moving within dendrites (yellow arrows), two static fNDs located outside dendritic membranes (blue arrows) and nine fNDs superimposed on dendritic membranes but not moving (gray arrows). The cell soma associated with the main dendrite is on the left (not shown). During the two-minute movie, two fND-containing endosomes, labeled #1 and #2 (in the box outlined with a solid white line) were observed moving towards the cell soma, in the same dendrite (retrograde transport). Right: superimposition onto a bright-field image of the positions of the two fNDs, determined by particle tracking, with a persistence of 10 s, at different times. Scale bars: 5 μ m and 1 μ m respectively. (c) A cartoon of our system showing the fND entrapped inside a cargo that travel along microtubule. Kymograph of the motion of fNDs #1 and #2 shown in (a) (white box), illustrating their "stop-and-go" behavior. Scale bars: 15 μ m (space) and 10 s (time).

2.4.4 Conclusion 2.4

In this section, we detailed our experimental strategy to perform dynamic study of intracellular transport inside branches of living neurons. We started with the choice of the biological material, namely

primary culture of hippocampal neurons, then the optical setup was adapted so that live cell imaging with optimal signal-to-background ratio can be performed. Finally, the most difficult part was to find a way to deliver fNDs into neurons. We detailed three different strategies and finally converged toward one utilizing the natural endocytosis pathway. Altogether, this allowed us to observe binary STOP&GO motion inside freely-developing branches of living neurons.

2.5 Conclusion of Chapter 2

Previous attempts to quantify intraneuronal transport have been based on cargo labelling with genetic constructs of chimeric fluorescent proteins. Limitations of this strategy are low transfection yield, uncontrolled protein overproduction, photobleaching of the reporters, and substantial cytotoxicity. QDots have been widely used to probe a variety of cellular events(Pinaud et al., 2010), owing to their high photoluminescence yield and good photostability. However, attempts to use QDs to study intraneuronal transport have been limited to the specific model of long axons of dorsal root neurons cultured in microfluidic devices. Moreover, the toxicity of QDs constitutive elements potentially limits their use in long-lasting studies and QDs blinking impedes high spatiotemporal resolution tracking. Considering these intrinsic limitations, there is a need for developing new generic approaches able to screen the functional impact of brain disease-related genetic modifications.

fNDs are emerging as versatile tools for both *in vitro* and *in vivo* bioimaging, taking advantages of their perfectly photostable emission in the far red, their high brightness and their absence of cytotoxicity. We have detailed our experimental strategy to perform dynamical study of the intraneuronal transport inside branches of living hippocampal neurons dissociated from mouse embryos, leading to the monitoring of the well-known binary STOP&GO motion.

However, for precise spatiotemporal quantification of this active transport, we will first have to extract the fND-labeled endosome trajectories from the videos using single-particle-tracking processing. But what can be the best approach to quickly and accurately perform this task? Moreover, as the "stop" and "go" phases of motion display specific spatiotemporal features, how can we automatically extract these two features to quantify them separately in space and time? Eventually, is our fNDs-tracking assay stable and sensitive enough to tackle relevant neurobiological questions?

CHAPITER 3

Spatiotemporal quantitative readouts and validation of the nanodiamond-tracking assay

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3.1 Introduction

At the end of Chapter 2, we showed that bare fNDs are easily endocytosed by living hippocampal neurons dissociated from mouse embryos. A large amount of trajectories can be recorded by this approach.

However, we now need to automatically extract trajectories and define relevant intraneuronal transport parameters.

This third chapter is dedicated to the quantitative analysis of fNDs trajectories and the validation of the single particle tracking (SPT) assay. We first discuss the choice of the SPT software and the measurement of the localisation precision of our live-cell imaging setup. Then, we review common SPT analysis tools used to study biological processes and we detail how we automatically detect the STOP and GO phases of motion, resulting in a set of eight transport readouts. We then describe how we have validated the "fND tracking assay" by demonstrating the microtubule-dependent transport of fNDs as well as the intra and inter-experiment stability of this approach. Finally, we conclude with some achievement this technique made possible.

3.2 Tracking of fluorescent nanodiamonds

Several microscopy techniques can be used to study biological processes at the molecular scale. Fluorescence Correlation Spectroscopy (FCS) or Fluorescence Recovery After Photobleaching (FRAP) are powerful approaches to study the motion dynamics of an ensemble of molecules inside living cells. However, only SPT approach allows direct visualization of the trajectory of a single fluorescent probe. First proposed in the late 1980's, SPT is of key importance for molecular scale quantitative analysis of intracellular motion processes. However, manually detecting and following a large number of individual particles are impossible and automated computational methods have been developed to perform such a tasks. Therefore, in this section, we first detail the principles underlying SPT. Then, we present the tracking software we used. Finally, we describe the calculation of the localisation precision of our live-cell imaging setup.

3.2.1 Precise and accurate localisation of single emitters

Conventional fluorescence light microscopies, such as confocal or two-photon microscopy, are widely used to study subcellular organisation and dynamic of proteins. However, as demonstrated by Abbe Ernst in the late 19th century, optical microscopy resolution is fundamentally limited by diffraction (to a spatial resolution of about 200 nm), meaning that light originating from a point source (e.g., one fluorescent molecule) in the object plane cannot be re-focused in the image plane as a single point. Both the wave property of light and the spatial filtering of the microscope circular aperture create an intensity distribution called the point spread function (PSF) which is the mathematical shape of the Airy pattern (see equation 3.1 and Figure 3.1a). Indeed, when light goes through a circular aperture (e.g. the microscope objective), the intensity of the diffraction pattern on a screen located far from the circular aperture (e.g. the camera) is given by:

$$I_{\text{Airy}}(\theta) = I_a \left(\frac{2J_1(kr\sin\theta)}{kr\sin\theta}\right)^2 = I_a \left(\frac{2J_1(x)}{x}\right)^2 \quad , \quad (3.1)$$

where I_a is the maximum intensity at the center of the pattern, J_1 is the Bessel function of the first kind of order one, $k=2\pi/\lambda$ is the wavenumber, r is the radius of the aperture, and θ is the angle of observation, i.e. the angle between the axis of the circular aperture and the line between aperture center and observation point. There is no simple analytical expression of the standard deviation of the Airy disk. A common approximation consists in ignoring the outer bright rings and approximate the first lobe by a Gaussian function as follow (in 1D):

$$I_{\text{Gauss}}(x) = I_a \exp\left(-\frac{x^2}{2\sigma^2}\right)$$
, (3.2)

where *x* represents the radial distance from the center of the pattern, and σ is the standard deviation. Here, σ is adjusted so that we obtain the optimal approximation to the Airy pattern, which leads to $\sigma \approx R_a/3$, where R_a is the radius of the first dark ring (Figure 3.1a).



Figure 3.1: From PSF fitting to trajectory registration. (a) Gaussian approximation of the PSF Airy disk. **(b)** Single-molecule image on an array detector fitted by a Gaussian to pinpoint the center of emission, which is further represented as a "localised data point" by a narrower Gaussian function (of sub-pixel size width). From PALM Zeiss web site. **(c)** Localisation precision and accuracy. From (Deschout et al., 2014) **(d)** Issue of linking detected spot in the case of trajectory crossings.

Due to diffraction, two objects very close to each other may not be distinguished as their PSFs sum up. Therefore, a resolution limit has to be defined. As for, the Rayleigh's resolution criteria states that these two objects can be optically separated if the maximum of the second emitter is located at a distance from the first emitter center larger than the position of the first minimum. On can show that this criteria yields a smallest distance at which two PSF of the same brightness can be resolved equal to 0.61λ / NA, where λ is the wavelength of light and NA is the numerical aperture of the microscope objective. In our case, using 100x objective with NA=1.49 and an emission wavelength of fNDs of λ =680 nm, the resolution limit is approximately 280 nm. To go further, please refer to recent reviews on the diffraction limit (Carlton, 2008; Schermelleh et al., 2010).

Although any single fluorescent probe will appear as a diffraction-limited spot through an optical microscope, its center coordinates can be determined with higher precision. This concept, at the heart of SPT, was stated a long time ago (McCutchen, 1967), but the first proof-of-principle came only in the 1980's where technological achievement for light sensitive detectors have made it possible to perform the really first SPT experiment. This technique have been largely used for the study of receptor diffusion on cell surfaces at this
time. Pioneered by W.W. Webb (Barak, 1981) who realized the tracking of biomolecules coupled to an organic fluorescent dye, Kusumi was then the first to perform the tracking of gold nanoparticle-coupled membrane receptor using light diffraction (Kusumi et al., 1993). Then, Triller and Dahan's breakthrough was to track single QDot-coupled membrane receptor in the synaptic cleft (Dahan et al., 2003).

The biological insights that can be gain with the application of single molecules or single particle tracking and localisation-based super-resolution nanoscopy is strongly dependant on the precision and accuracy at which single fluorescent probe can be localized. For example, in the late 1990's, a lot of studies reported anomalous subdiffusion behavior. Based on SPT of membrane bound particles, researchers have suggested that anomalous subdiffusion is a ubiquitous process in biology. However, it was shown that this phenomenon was actually inherent to SPT approach having weak localisation accuracy (Martin et al., 2002).

Therefore, it is mandatory to fully understand the concepts of **localization precision** and **accuracy** of single emitters. Let us assume the ground truth position of a fluorophore and the multiple recordings of its position using an optical setup (Figure 3.1c). The localisation precision corresponds to the spread of the multiple detections of the fluorophore positions around its mean value. It is usually calculated as the standard deviation or the Full Width Half Maximum (FWHM) of this dispersion. Conversely, the localisation accuracy measures the difference between this mean value and the ground truth value, which cannot be experimentally measured. Consequently, these two parameters are independent and can be impacted by different parameters, such as emitter properties (e.g. single-dipole emitters) or experimental factors (e.g. sample background, drift during detector exposure...) (Deschout et al., 2014). Many research groups have tried to theoretically describe the localization precision or accuracy of a single fluorescent probe. The reader can refer to the following reviews for more details (Deschout et al., 2014; Small en Stahlheber, 2014).

Practically, fluorescence emission is recorded by photoelectric conversion-based device such as Electron Multiplying Charge Coupled Device (EMCCD) array detector or Avalanche photodiode (APD) point detector. Image is constituted by the signal of the fluorescent probe and some background and noise. The **localisation precision** was theoretically described by the following equation (Thompson et al., 2002):

$$\sigma \equiv \langle (\Delta x)^2 \rangle \approx \sqrt{\frac{s^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s^4 \sigma_B^2}{a^2 N^2}} \quad , \quad (3.3)$$

where *s* is the PSF standard deviation, *N* is the number of detected photons per exposure time, *a* is the pixel size, and σ_B the background standard deviation. Two sources of noise are present in this equation: (i) photon shot noise varying as $N^{-1/2}$, and which corresponds to the standard deviation of the photon distribution detected by one pixel (Poisson distribution) (first term in equation 3.3) and (ii) background noise varying as N^{-1} , and which is created by out-of-focus fluorescence, detector readout noise, dark counts, auto-fluorescence and other factors (third term in eq. 3.3). Moreover, we have to take into account the pixelation noise related to the uncertainty of where the photon arrives within the pixel and which is easily calculated as the standard deviation of a top-hat function (second term in eq. 3.3). At high light level, the shot noise is the dominant term of the localisation precision formula, while at low light level it is the background noise.

However, in practical situations, depending on the acquisition procedure, external perturbations, such as mechanical vibrations, thermal and mechanical drifts, should also be considered. Therefore, as shown in Figure 3.2, the experimental value of the localisation precision will be always larger than the theoretical limit of eq. 3.3.

3.2.2 Choice of the Single Particle Tracking software

A plethora of parameters can affect the robustness of single-particle tracking: the dynamics of the biological process, the density and signal of tracked particles, but also non-uniform background (like in cell), spot shape and size (varying between images due to defocusing), and photobleaching. A time-lapse image sequence often contains a high number of particles so that manual annotation is not feasible, and computer algorithms are compulsory to perform this tedious task.

SPT need two distinct steps for trajectory reconstruction: (i) **spot detection** and (ii) **spot linking** (Figure 3.1b, d). Therefore, tracking software can be classified with respect to the spot detection algorithm and to the trajectory reconstruction algorithm. Spot detection can be done using either a Gaussian fitting or a wavelet fitting (for the most recent and widely used approaches). The latter was shown to be one order of magnitude faster than the former (Izeddin et al., 2012). Then, two classes of tracking algorithm exist to do trajectory reconstruction: deterministic tracking and probabilistic tracking. The former performs spot detection and linking at the same time, through a nearest-neighbouring algorithm. The latter is based on spot detection first and once the entire stack has been processed, an algorithm based on a Bayesian inference approach links the spots with each other. The second strategy is more robust as it can easily overcome the issue of crossing particles (Figure 3.1d) (Godinez et al., 2009).

An objective comparison of particle tracking methods has been done by all the leading groups in this field (Chenouard et al., 2014). Although no single method performed best whatever the experimental configuration is, the results revealed clear differences between the various approaches. In particular, the authors concluded that all methods perform well for sufficiently high signal-to-noise ratio (SNR \geq 5).

Therefore, we decided to process our data with **ICY Particle Tracking plugin** (GPLv3 open source) developed by the Quantitative Image Analysis Unit at Institut Pasteur (Paris, France) (Chenouard et al., 2013). Interestingly, although the objective comparison study came out after we started this experiment, we were glad to see that our choice was relevant. Indeed, our specifications are as follow: medium fNDs density per field of view (intra and extracellular), high SNR (>4), alternation between two motion types (directed and brownian), 2D+time image acquisition, and dynamic fNDs intersecting with static fNDs (on the extracellular membrane). For these criteria, the authors of (Chenouard et al., 2014) showed that ICY Particle Tracking plugin is the most efficient one. This plugin is also performing the best for most of the criteria evaluated during this challenge.

The strength of this plugin is that it allows automatized trajectories extraction of multiples objects simultaneously in a 3D image sequence (x,y,t), where the number of targets can vary through time (objects

can appear and disappear). Also, false detections (not originating from a target) are automatically detected and discarded. Moreover, as it is based on Bayesian inference approach, different dynamical processes can be considered (diffusive, directed, or both). Eventually, the probabilistic tracking method is based on a 'multiframe' approach in the sense that the best set of tracks is determined within multiple past and future frames.

3.2.3 Measurement of the live-cell imaging setup localisation precision

The quantitative analysis of trajectories depends on SPT experimental localization precision. The latter can be affected trivially by mechanical vibration or thermal fluctuations. The first ones are drastically reduced by installing the microscope on an anti-vibration optical table system. Since thermal fluctuations can induce drift during the course of video acquisition, we have enclosed the whole imaging setup inside a chamber thermalized at a controlled temperature of $37.0\pm0.1^{\circ}$ C. The remaining fluctuations most likely originate from residual mechanical vibrations.

We measured the localization precision by considering a sample of fNDs immobilized by spin-coating an aqueous suspension of the nanoparticles onto a coverslip and recording a video of n=200 frames with the same acquisition parameters (20 frames per second, 35 MHz camera reading frequency, pre-amplifier setting: 3.8 and EM gain: 50) and in the same conditions as for live-cell imaging (37°C and water-saturated air flow with a partial pressure of 5% for CO₂). FNDs tracking was done using the same tracking software parameters as routinely performed. Then, we obtained the positions of about 180 fNDs having different fluorescence intensities covering a range comparable to the one encountered during the tracking of fND entrapped in endosomes. Then, we quantified the localization precision based on the standard deviation of all recorded coordinates for each fND independently.

For a given fND, the localization precision is described by the spread of its coordinates x_i estimated n times (n=200 here) and is commonly expressed in terms of standard deviation σ_x (respectively for σ_y):

$$\sigma_x = \sqrt{\frac{1}{n-1} \sum_{i=1}^n (x_i - \bar{x})^2}$$
 , (3.4)

Another representation of the localization precision is based on the mean squared displacement. Indeed, the spin-coated fNDs can be seen as molecules undergoing a motion within a confined area (see section 3.3.1) of size L inferred from the MSD fit with respect to time using the following equation (Kusumi et al., 1993):

$$MSD(t) = \frac{L^2}{3} \left(1 - \exp\left(-\frac{12Dt}{L^2}\right) \right) , \quad (3.5)$$

where $L^2 = \pi R^2$ is the confined area in which the motion is restricted and *D* is the diffusion coefficient.

Then, we plotted these two representations of the localization precision with respect to **signal-to-noise ratio** (SNR) (Figure 3.2a, red and blue curves respectively), where SNR was determined as follow:

$$SNR = \frac{F - F_0}{\sigma} = \frac{F - F_0}{\sqrt{F + \sigma_B^2}}$$
, (3.6)

where *F* is the maximum fluorescence level, F_0 is the background level, and σ is the standard deviation of the noise, which is calculated from the photon shot-noise and the background noise σ_B by: $\sigma^2 = F + \sigma_B^2$. For this SNR measurement, we converted all the parameters from Analog-to-Digital unit (ADU) into photon number N_{photons} , using the following equation:

$$N_{\rm photons} = \frac{(Counts_{\rm ADU} - BaseLevel_{\rm ADU}) * sensitivity}{EM_{\rm gain}.QE} \quad , \quad (3.7)$$

where, according to our Andor iXon+ DU885 EMCCD array detector specifications, at 35 MHz and 3.8x preamp setting, the mean *BaseLevel* is equal to 394 ADU, the sensitivity is 1.5 electron/ADU, the EM_{gain} is 30 and the quantum yield QE at 700 nm is 0.65.

We finally compared these experimental curves with the theoretical one described by equation (3.3) (Figure 3.2a in grey). Here, the standard deviation of the PSF was experimentally measured to be $s=100.9\pm0.7$ nm (mean±SDev), which is consistent with the expected value of diffraction limited imaging. Then, the pixel size *a* is of 80 nm, σ_B corresponds to the standard deviations of F_0 measured over the 200 frames and the total number of detected photon *N* is equal to the volume of the 2D-gaussian function $N = 2\pi(F - F_0) \cdot \left(\frac{s}{a}\right)^2$. Therefore, in the case of one of the brightest fND, we had F=15,726 ADU counts, $F_0=816$ ADU counts leading to SNR=31 and N=11,500 photons. The theoretical limit of the localization precision precision (deduced from equation 3.3) associated to these values is 1.5 nm using fNDs containing 15 NV color-center per particle imaged at 50 ms integration time per frame. These values are comparable to the ones published by (Yildiz, 2003), who have imaged a single cyanine 5 dye with 500 ms integration time and have obtained a SNR=32 and N=14,000 photons and achieved a localization precision of 1.2 nm. This comparison shows that we have gained one order of magnitude in temporal resolution thanks to the high brightness of fNDs.

Figure 3.2a displays the localization precision vs SNR. We observed that both the experimental curves have a similar shape than the theoretical calculation (all of them fitted by an exponential function as guides for the eyes), with the experimental ones shifted up by several tens of nanometers (from 1.5 nm (grey), 8.2 nm (red) to 18.4 nm (blue)). As already mentioned, this is because the theoretical equation does not take into account the environmental perturbation but only the electronic, photon and background noise on the camera array detector.

However, for the rest of the study, we used a more operational definition to evaluate the brightness of the spot, namely the signal-to-background ratio (SBR), defined as follow:

$$SBR = \frac{F - F_0}{F_0}$$
 , (3.8)

where F is the maximum fluorescence level and F_0 is the surrounding background fluorescence level, both of them in ADU counts unit. The theoretical and the experimental localization precision can also be plotted with

respect to SBR as shown in Figure 3.2b, and we have observed that these curves have a similar shape than the ones of Figure 3.2a.

According to Figure 3.2b, to achieve a localization precision better than 30 nm as reported in the field of SPT (Mudrakola et al., 2009) we need to discard spots having a SBR<3. According to Figure 3.2a, the same precision of localization if obtain for a signal-to-noise ratio SNR \approx 10. Practically, for the quantification of transport parameters we did not include trajectories for which more than 30% of their points had a SBR smaller than 3. For a given experiment, this represent a fraction of about 13±2% (mean±s.e.m.) of the trajectories that are not included in the final calculation of the intraneuronal transport parameters. Incidentally, the measurement range of the endosome velocity is therefore limited on its lower bound by the setup spatial (30 nm) and temporal (50 ms) resolution to about 600 nm/s.



Figure 3.2: Theoretical and experimental localisation precision of the live cell imaging set-up as measured using immobile fND spin-coated onto a coverslip. (a) Localisation precision with respect to the signal-to-noise ratio (defined in eq. 3.6) inferred from the theoretical limit of equation (3.3) (in grey) and from measurements of the fluorescence and background intensities at each fND location (standard deviation [eq. 3.4] in red and MSD confinement radius in blue [eq. 3.5]). (b) Localisation precision vs the signal-to-background ratio as defined in eq. 3.8 for both the experimental value (standard deviation, in red) and theoretical value (grey).

3.2.4 Conclusion 3.2

In this section, we detailed the basics of the SPT method, from the PSF fitting to the concept of localisation precision. Then, we saw that open-access "particle tracker" plugin from ICY offers the best processing time/accuracy trade-off. Finally, we described the calibration of our live cell imaging setup, leading to less than 30 nm when restricted to fNDs with a SBR>3. However, at this point, only the coordinate sequences of the position of each tracked object at each time point have been extracted. Therefore, how can we automatically extract and quantify fNDs transition rates from these single-molecule tracking data?

3.3 Spatiotemporal quantitative measurements of the STOP&GO

binary motion

Dynamic parameters such as velocity, pausing time or diffusion coefficient can be assessed in various ways. For example, the two first readouts can be evaluated by auto and cross-correlation of neighbouring images (Welzel et al., 2009), which does not require any single particle tracking. However, contrary to SPT, this approach cannot reveal the full complexity and heterogeneity at a single trajectory level, thus providing limited throughputs.

Consequently, to extract intracellular transition rates from single-molecule tracking data, standard methods such as mean squared displacement (MSD) and kymograph have been used (Ehrensperger et al., 2007). Therefore, in this section, we describe both approaches and discussed their limitations when dealing with precise spatiotemporal description of the STOP&GO binary motion. Then, we present the specific tools that we developed to automatically measure the intraneuronal transport parameters. Finally, we explore the spatial distribution during a STOP phase.

3.3.1 Common SPT analysis tools to study dynamic processes

Several basic mathematical tools can be applied to extract relevant features of a trajectory and gain more insight into intracellular dynamics (Table 3.1). For example, geometrical tools such as directional change can inform on the back-and-forth movement of cargos undergoing tug-of-war motion (Hancock, 2014), or switching from one to another microtubule or to another neuronal branch. Others more sophisticated automated approaches have been developed to characterize trajectories. The two most common tools are the **mean-square-displacement** (MSD) and the **kymograph**.

The most commonly used SPT analysis tool is the **MSD**. This easily computed function is performed to determine the mode of displacement of particles over time. Its measurement can give access to the diffusion coefficient *D* and the velocity of directed motion (Kusumi et al., 1993; Qian et al., 1991).

$$MSD(n\Delta t) = \frac{1}{N} \sum_{i=1}^{N} ((x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2) \quad , \quad (3.4)$$

where *N* is the total number of frames, Δt is the time interval between two consecutive frames, and (x_i ; y_i) are the positions on the *i*th frame.



Measure	Definition
Total distance traveled	$d_{\text{tot}} = \sum_{i=1}^{N-1} d(\mathbf{p}_{i}, \mathbf{p}_{i+1})$
Net distance traveled	$d_{\rm net} = d(\mathbf{p}_1, \mathbf{p}_N)$
Maximum distance traveled	$d_{\max} = \max_i d(\mathbf{p}_1, \mathbf{p}_i)$
Total trajectory time	$t_{\rm tot} = (N-1)\Delta t$
Confinement ratio	$r_{\rm con} = d_{\rm net}/d_{\rm tot}$
Instantaneous angle	$\alpha_i = \arctan(\gamma_{i+1} - \gamma_i) / (x_{i+1} - x_i)$
Directional change	$\gamma_i = \alpha_i - \alpha_{i-1}$
Instantaneous speed	$v_i = d(\mathbf{p}_i, \mathbf{p}_{i_1+1})/\Delta t$
Mean curvilinear speed	$\bar{\mathbf{v}} = \frac{1}{N-1} \sum_{i=1}^{N-1} \mathbf{v}_i$
Mean straight-line speed	$v_{\rm lin} = d_{\rm net}/t_{\rm tot}$
Linearity of forward progression	$\eta_{ m in} = v_{ m lin}/\overline{v}$
Mean squared displacement	$MSD(n) = \frac{1}{N-n} \sum_{i=1}^{N-n} d^2 \left(\mathbf{p}_i, \mathbf{p}_{i+n} \right)$

Table 3.1: Basic quantitative measurements applicable to describe particle dynamics. From (Meijering et al., 2012)

Three distinct modes of motion can be depicted on the basis of the MSD measurement versus lagging time (Figure 3.3):

- **Simple diffusion mode** (Figure 3.3 (i)): the particle undergoes simple Brownian diffusion. MSD varies linearly with the time with a slope nD (n=4 or n=6 in 2D or 3D). MSD(t) = 4D.t in 2D.

- **Directed diffusion mode** (transport mode) (Figure 3.3 (ii)): the particle moves in one direction at a constant velocity with superimposed random diffusion with a diffusion coefficient *D*. The MSD varies as a parabola with a differential coefficient of 4*D* at time 0 (initial slope): $MSD(t) = 4D.t + v^2.t^2$

- **Restricted diffusion mode** (Figure 3.3 (iii) and equation 3.6): the particle undergoes Brownian diffusion within a confined area of size *L*. Here, the curve at the infinity can be approximated by a horizontal asymptote corresponding to $MSD=L^2/3$ in each dimension (Kusumi et al 1993).

Another way to perform motion classification is to measure the α -coefficient of the power law curve MSD(t), as: log[MSD(Δt)]= α log(Δt)+Cte. Therefore, anomalous diffusion, pure Brownian diffusion and directed diffusion motion will be assigned with respect to $\alpha < 1$, $\alpha = 1$, and $\alpha > 1$, respectively.



Figure 3.3: MSD calculation for the study of molecular dynamics. (a) Typical trajectories of gold particles attached to E-cadherin membrane proteins. **(b)** MSD plots as a function of time t for the particles in (a) with the following motion mode: (i) Brownian diffusion, (ii) Directed diffusion and (iii) Confined diffusion. From (Kusumi et al., 1993)

MSD can be instrumental to perform lateral diffusion measurement of QDot-targeted membrane receptors of synaptic and peri-synaptic part (Triller en Choquet, 2008). In the case of intracellular tracking, MSD calculation has been perform to infer dynamic characteristic of QDots-targeted and SWNT-targeted kinesin motion for example (Courty et al., 2006; Fakhri et al., 2014; Sun et al., 2014). Figure 3.4a shows an example of MSD calculation to measure both diffusion coefficient of fixed and moving particle and the velocity of the latter.

However, MSD analysis is not robust enough when dealing with short or long trajectories which contain both diffusive and directed parts (Meijering et al., 2012). Moreover, this measurement requires a minimum of ten points as the inaccuracy of diffusion coefficient measurement increases when the length of trajectory decreases (Weimann et al., 2013). Therefore, it is not sufficiently precise to use MSD analysis to automatically detect the two states of motion.

Another widely used SPT analysis tool is the **kymograph** (Chenouard et al., 2010; Zhang et al., 2011). It is a powerful 2D representation of spatial and temporal information. Usually, the horizontal axis corresponds to the spatial coordinate along this path, while the vertical axis corresponds to the time frame in the image sequence. Hence, the pixel value at position (s, t_n) in the kymograph image corresponds to the pixel intensity at position s along the extraction path at frame n of the sequence. Kymograph can give access to several parameters and can be processed simultaneously on a lot of trajectories only if they are all moving in a single direction (Figure 3.4b). This approach can be easily performed for trafficking study in axon if they are enforced to develop in only one direction using a microfluidic device (Mudrakola et al., 2009; Zala et al., 2013).



Figure 3.4: Example of MSD and kymograph analysis of single molecule dynamics. (a) Typical trajectories of linear motions of QDs-labelled kinesin and random motions of PEG-QDs, together with their associated MSD plotted vs time. From (Sun et al., 2014). (b) Forward and backward displacement analysis of fast axonal transport using kymograph. Yellow stars show biased measurement. From (Zala et al., 2013).

However, it will not be suitable for our analysis where freely developing neuronal brnaches show random isotropic morphology. Moreover, user definition of the path of interest is necessary to get a more reliable outcome, which makes kymograph analysis slow and tedious when dealing with biological question requiring high statistical power. Besides, fine and robust spatiotemporal parameters of the trajectory cannot be extracted. When kymograph is used to tackle biological question, it only consider velocity (forward and backward), run length and/or pausing time but no other readouts (Courty et al., 2006; McKenney et al., 2014; Zala et al., 2013). An example from recent literature is shown in Figure 3.4b. In this case, readouts can be biased due to the smoothing algorithm isolating straight segments. Indeed, anterograde and retrograde velocity are extracted from green and red segment respectively, while static motion is depicted in blue. Herein, the yellow star shows a region where three consecutive stop and go motions are considered as a single anterograde segment, resulting in an underestimation of the velocity.

Altogether, the MSD and kymograph drawbacks that we highlighted stress the need for developing a new SPT analysis tool that provide a more precise and unbiased quantitative description of transient dynamics of the intraneuronal transport.

3.3.2 Spatiotemporal detection of STOP&GO binary motion

Custom-made algorithm for the detection of STOP&GO motion

The **confinement ratio** (CR), also known as meandering index or straightness index, is often used for cell tracking, such as immune cells (Beltman et al., 2009). CR corresponds to the ratio of the vectorial

displacement over the total travelled length (Figure 3.5a). Therefore, CR varies between 0 (perfectly confined motion) and 1 (perfectly directed motion). However, CR is useless if the full trajectory is taken into account because it will tend to zero. Therefore, as we want a local knowledge of the particle dynamic and not the entire one, the use of a sliding window of few consecutive frames is a perfect trade-off.



Figure 3.5: Detection of the "stop-and-go" motion phases. (a) Definition of the confinement ratio for the first coarse detection. **(b)** Application to trajectory#2 of Figure 2.16 (Chapter 2). Green and blue stars referred to short GO and STOP phase respectively. **(c)** Kymograph of trajectory#1 and #2 showing green and blue arrow heads pointing out the same position as stars in (b). These events are usually missing when kymograph is used to infer velocity, run length and pausing time, leading to erroneous measurements. **(d)** "Short stop" filter. **(e)** "Directional change" filter. **(f)** "False positive" filter. Scale bar for (e-f): 100 nm, 50 nm and 100 nm respectively.

Here, the confinement ratio function R_{conf} is calculated for each trajectory point, using a forward sliding window of four consecutive points (empirically determined), as the relative distance d_{rel} between extreme positions *i*=1 and *i*=4 over the absolute distance Σd_i (*i*=1,...,4). R_{conf} is used to discriminate between static ("**stop**") and directed ("**go**") phases of motion, where $R_{conf} \approx 1$ corresponds to an endosome in a directed motion "go" state, and $R_{conf} \approx 0$ corresponds to the "stop" state. A graphical representation of R_{conf} function applied to trajectory #2 (from Chapter 2 Figure 2.17b, c) is shown in Figure 3.5b. In the trajectory analysis algorithm, all the trajectory points are first initialized to the "stop" state. Then, a "go" state is assigned to each trajectory point based on its R_{conf} value which have to be larger than a cutoff fixed at $R_{conf}^{cutoff}=0.9$ (horizontal dashed black line). As in figure 3.5b, the R_{conf} function reveals the presence of these two phases, with a histogram for R_{conf} distribution on the right. This result is in agreement with the one given by the kymograph #2 of the same trajectory depicted in Figure 3.5c. However, the two-state motion discrimination using the confinement ratio is more accurate than with the kymograph as shown by the stars and arrow heads (blue and green for STOP and GO respectively) pointing to the same events in R_{conf} and kymograph representation respectively.

Then, we sequentially apply three different filters to refine the detection of "stop" phases (Figure 3.5df). The first filter, namely "short stop", is based on the localisation precision through the calibration curve in Figure 3.2. If two consecutive "go" state positions (depicted in green) are separated by a distance smaller than the localization precision of 30 nm, the first is considered to be a "stop" (Figure 3.5d). This implies that a velocity cut-off is set at 30/0.05=600 nm/s. The second filter, namely "directional change", is based on the angle between two consecutive segments where points are "go" tagged. If this angle is larger than 80° (empirically determined), the point separating the two segments is considered to correspond to a "stop" (Figure 3.5e). Finally, the third filter, namely "false positive", is based on the detection of a point identified as a "go" which is within a "stop" region. This point is considered to be a false positive and is reassigned as a "stop".



Figure 3.6: STOP&GO motion and molecular landscape. (a) Examples of a color-coded trajectory displaying STOP (blue) and GO (green) spatially and temporally. Scale bars: 500 nm and 1 s respectively (b) Possible interpretations of short pausing and long pausing due to microtubule crossings or hindrance close to dendritic spines position. Adapted from (Bálint et al., 2013).

Altogether, this procedure leads to a precise colour code of trajectories as shown in Figure 3.6a. Before going further, some qualitative observations can be done. Indeed, the presence of short pauses and long pauses separated by directed motion is quite intriguing. These alternation of dynamical processes might be due to the presence of a particular molecular landscape encountered by the moving cargo, such as roadblocks, traffic jams...(Lakadamyali, 2014). Another explanation might be that cargos have to deal with different 3D spatial organisations of microtubules or particular hindrance at the basis of a dendritic spine (Figure 3.6). Indeed, it was shown that microtubules cross each other (Bálint et al., 2013) and at this crossing location, the cargo can behave differently. The cargo does not stop if no microtubule is beneath, but if there is a microtubule above, it can be squeezed and even can detach if the distance between the two microtubule is less than ≈ 100 nm. It would be interesting to unravel the processes responsible for such a cargo behaviors by combining microtubule imaging (with 3D super-resolution microscopy) and cargo SPT in a dual-view manner.

Readouts measurements and spatial description of "stop" phase

Based on this color-coded trajectory, the different "stop-and-go" phases of the fNDs entrapped in cargos are registered and we are now able to quantify them in both space and time, leading to eight intraneuronal transport readouts, namely velocity (in μ m/s), processivity (in seconds), run length (in μ m), pausing time (in seconds), pausing frequency (events per minute), longitudinal and perpendicular displacements (in nanometers), and the diffusion coefficient (in μ m²/s). Mean values and other statistical properties of these parameters for wild-type embryos are provided in Table 3.2.

Readouts	Range	Median	Mean	Standard Deviation	Interquartile Range
Velocity (µm/s)	0.66 - 3.75	1.31	1.45	0.54	1.07 - 1.64
Processivity (s)	0.14 - 6.4	0.48	0.64	0.6	0.33 - 0.72
Run Length (μm)	0.19 – 15.54	0.93	1.5	1.98	0.56 - 1.63
Pausing Time (s)	0.05 – 9.78	0.32	0.48	1.04	0.18 - 0.53
Pausing Frequency (events/min)	5.2 – 129.3	64.1	64.5	24.6	48.1 - 78.5
Perpendicular Displacement (nm)	10.2 - 259	55.4	63.1	33.6	42.1 - 76
Longitudinal Displacement (nm)	34.4 - 644	168.8	183.5	80.37	133.9 - 209.2
Diffusion Coefficient (µm²/s)	0.001 - 0.071	0.016	0.020	0.015	0.009 - 0.037
Signal-to-Noise Ratio	4.5 - 61.3	14.8	17.1	9.5	10.1 - 21.6

Table 3.2: Intraneuronal transport readouts statistics. All trafficking parameters were extracted from n=460 trajectories acquired from n=11 C57Bl6 (wild type) mouse embryos. The range and interquartile range correspond to the 0-100% and 25-75% percentiles, respectively.

The **velocity** *v* was calculated as the length of the displacement vector over time from the first to the last point of a "go" phase, whereas the curvilinear distance and time separating these two points corresponded

to **run length** and **processivity**, respectively. The **diffusion coefficient** *D* was calculated during the "go" phase, by fitting the mean-square displacement (MSD) to the quadratic equation $MSD(\Delta t) = v^2 \Delta t^2 + 4D\Delta t$. According to the Stokes-Einstein law, *D* is inversely proportional to intracellular viscosity. During a "stop" phase, the **pausing time** was defined as the duration from the first to the last point, the **pausing frequency** was the number of "stop" phases per minute, and the **longitudinal** and **perpendicular displacements** were calculated as described in Figure 3.7.

Briefly, these two parameters were calculated as follow (Figure 3.7a): for each STOP phase, a straight line (Δ) was drawn between the first STOP and the next first GO with its director vector **u**. Consequently, the line (Δ^{\perp}) is perpendicular to (Δ) and is defined by its normal vector **n** (equation 3.7 and 3.8). Then, algebraic projections D_{i_n} and D_{i_u} on the straight line (Δ) were calculated for each point *i* of this STOP phase (equation 3.9 and 3.10). For both directions, amplitudes of the displacement were calculated and minor and major axis were assigned accordingly.

(
$$\Delta$$
): $Ax - y + B = 0$, (3.7) and (Δ^{\perp}): $x + Ay - C = 0$, (3.8)

$$D_n^i = \frac{Ax_i - y_i + B}{\sqrt{1 + A^2}}$$
, (3.9) and $D_u^i = \frac{x_i + Ay_i - C}{\sqrt{1 + A^2}}$, (3.10)

Interestingly, we observed that the "stop"-phase positions were located within an ellipse (Figure 3.7b, c). Indeed, when longitudinal versus perpendicular displacement are plotted (n=4490 "stop" events extracted from n=460 trajectories acquired from n=11 *C57Bl6* mouse embryos), we observed a correlation around a line (dashed red) having a slope of 0.287 ± 0.003 (mean ± standard deviation) and a correlation factor R^2 = 0.55 which highlights the elliptical pattern of motion.

Thereafter, we also proved that the longest elliptical axis is aligned with the direction of the "go" motion most of the time (Figure 3.7d,e). A schematic diagram in Figure 3.7c shows the direction Δ_1 (Δ_2) of the trajectory before (and after) the "stop" phase which were determined as the linear fit to the last (or first) 3 points before (or after) the first (or last) "stop". θ_{output} is the "output" angle between Δ_2 and Δ_1 and $\theta_{ellipse}$ is the "ellipse" angle between Δ and Δ_1 . Therefore, the distribution of "output" angle was proved to be centered on $\theta_{output} = -0.10\pm0.65^{\circ}$, close to zero. This result suggests that microtubules are locally parallel to each other, presumably running along the shaft of the dendrite. Then, the distribution of "ellipse" angle was proved to be centered on $\theta_{ellipse} = 0.66\pm0.83^{\circ}$, close to zero. This result suggests that, during a "stop" phase, endosomes rarely change microtubules. Eventually, when θ_{output} and $\theta_{ellipse}$ are plotted against each other, two forbidden configurations occur when both θ_{output} and $\theta_{ellipse}$ display strong directional changes of opposite angle. However, we did not go further to explain this observation.

As already mentioned in the Chapter 2, one tracking experiment will lead to roughly 40 trajectories. Therefore, the next step is to find a way to treat this batch of trajectories to compare two or more different data sets related to different biological configuration. However, two main constraints have to be taken into account to avoid bias. First, because both STOP&GO motion states are measured in space and time, the extremities of each trajectory underestimate the parameter ground truth values (Figure 3.8a). Secondly, the SBR of each trajectory point has to be evaluated to ensure that 30 nm localization precision is reached at any time.



Figure 3.7: Elliptical quantification of "stop" phases and qualitative descriptions. (a) Geometrical references for perpendicular and longitudinal displacement calculations. (b) Plot of longitudinal versus perpendicular displacement proving the elliptical shape. Black line and dashed red line are the first bisector and the linear data fit respectively (c) Schematic diagram showing how references were chosen. (d) Distribution of "output" angle θ_{output} (e) Distribution of "ellipse" angle $\theta_{ellipse}$. (f) Dots plot of θ_{output} versus $\theta_{ellipse}$ showing two forbidden configurations.

Therefore, the first strategy we tried was to use the bootstrapping approach to artificially recreate a past and a future behavior based on the entire set of data. A randomization of the full data set was performed

using a random generation function and then the trajectories were concatenated all together. Then, considering the precision localization calibration curve, all trajectory segments having a SBR<3 were deleted. At the end, this procedure was repeated several times to be unbiased (Figure 3.8b).

However, each trajectory may reveal a unique neurobiological process, which can be different between two neurons. Even in the same neuron, we can expect different dynamics for each fND-containing cargos, as the type of cargos, the number of molecular motor recruited and even the landscape with dynamical rearrangements (obstacles, microtubule depolymerization...) can vary spatially and temporally. Consequently, to account for biological variability, we decided to extract the **readouts for each trajectory** independently, and did not apply any bootstraping. Blunt extremities were not included for the mean or median readouts calculation. Moreover, we did not consider short trajectories (less than 50 frames) as well as trajectories with more than 30 % of points imprecisely localized. Altogether, we obtained the statistical figures of intraneuronal trafficking readouts shown in Table 3.2, calculated from n=460 trajectories acquired from n=11 *C57Bl6* mouse embryos.



Figure 3.8: Batch treatment of extracted trajectories. (a) One trajectory at a time with exclusion of the first and the last events **(b)** Bootstrapping Stochastically Reconstructed Intraneuronal Trajectory. Briefly, all trajectories are first aligned with each other, then they are randomly concatenated and finally dim segments are detected and deleted. Readouts calculation is made and the all process is repeated one thousand times.

3.3.3 Conclusion 3.3

In this section, we described the most common SPT analysis tools, namely MSD and kymograph, which can be used to measure dynamical parameters such as diffusion coefficient, velocity or run length. However,

both have limitations that prevent the detection of fine intraneuronal transport dynamics. These observations prompted us to develop our own analysis approach based on the confinement ratio function, yielding eight distinct dynamic parameters. However, is our fNDs-tracking assay stable within the course of one experiment and between two independent experiments? What is its sensitivity? How can we demonstrate that we are recording microtubule-dependent transport?

3.4 Validation of the "nanodiamond-tracking assay"

When a novel methodology is developed, several validation tests have to be successfully passed. This is mainly the purpose of this section. First, we evaluate the stability during the course of one experiment (intrastability) and between two independent experiments (inter-stability). Then, we assess its sensitivity to nanomolar concentration of drugs that depolymerize either microtubules or actin. Eventually, we show that the fNDs-tracking assay can be instrumental to optically resolve microtubule bundles in living neurons.

3.4.1 Intra and inter-experiment stability of the method

The **intra-stability** of the fNTA was assessed to prove that this methodology does not affect the normal functioning of the neurons, due to cyto or photo-toxicity for example. To do so, we first evaluate the global variation of velocity and processivity over the entire course of the experiment (Figure 3.9a). The slope of both linear fits are -3.9×10^{-4} and 5.7×10^{-4} respectively, very close to 0, indicating no dependence of experimental procedure with time. Then we compared the eight transport parameters measured during the first and the last 15 min of the 45 min duration experimental runs for three independent experiments (Figure 3.9b, c). No statistically significant changes are observed, except for longitudinal displacement, for which *p*=0.03, indicating that this readout is not entirely reliable. Because both longitudinal and perpendicular displacement are linked throughout their calculation, we will not pay much attention on these two parameters for the rest of the study. Therefore, most of the trafficking readouts remained constant during the 45-minute recordings for each culture well.

Finally, the **inter-stability** of the technique was assessed for two distinct experiments (different cultures and different pregnant females). Figure 3.10 shows no significant differences between the readout values (even though longitudinal displacement tends to decrease). Altogether, these results demonstrate the good stability of the fNDs-tracking assay.

However, because both perpendicular and longitudinal displacements are intrinsically linked in the way we extract them, we decided to consider none of them to interpret the data elsewhere.



Figure 3.9: Intra-experiment stability of the method. (a) Variation of two readouts (mean values of velocity and processivity) for 64 consecutive trajectories over a total duration of 45 minutes acquired from a single hippocampal neuron culture well. The dashed black lines are linear fits to the data. (b) Comparison of the readouts between the first 15 minutes and the last 15 minutes of acquisition, for experiments carried out in triplicate. **(c)** Corresponding Wilcoxon U-test p-values.



Figure 3.10: Inter-experiment stability of the method. (a) Intraneuronal transport parameters for two different experiments: one well each from two cultures and two different pregnant females (n>30 trajectories from n=4 embryos each experiment). **(b)** Corresponding Wilcoxon U-test p-values.

3.4.2 Quantification of kinesin versus dynein trafficking parameters

Intracellular transport is fundamental for morphogenesis and biological function. It requires both molecular motors and "rail tracks" where long-range transport is performed by kinesin and dynein traveling along microtubule "tracks", while short-range transport is done by myosin traveling along filamentous actin (Hirokawa et al., 2009).

In some occasions, the fND-containing endosomes display directional changes, going back on themselves for short periods of time. These observations suggest that endosomes can change motor type, as previously reported for kinesin and dynein, which oppose each other in a sort of "tug-of-war" situation (Hancock, 2014). Therefore, this prompts us to measure intraneuronal transport parameters separately for kinesin (motion from the soma to the distal part) and dynein (motion from the distal part to the soma). In Figure 3.11a, given the uniform polarization of microtubules in the distal region, the retrograde motion of #1 is dynein-driven, whereas the anterograde motion of #2 is kinesin-driven. As shown in Figure 3.11b,c, no statistically significant changes were observed, except for longitudinal displacement, for which p=0.003, confirming that this readout is not entirely reliable, as already observed in intra-stability tests.



Figure 3.10: Quantification of kinesin versus dynein active transport parameters. (a) DIC image merged with maximum intensity projection of fND movie (red channel), superimposed to trajectories #1 and #2. Green arrows indicate the two different directions of motion. Scale bar: 5 μ m. (b) Intraneuronal trafficking readouts for kinesin versus dynein molecular motors (trajectories extracted from different experiments). (c) Wilcoxon U-test p-values for the eight readouts.

Altogether, these results allow us to pool all trajectories for each set of data, without worrying about the molecular motor recruited and regardless of the direction of motion. Therefore, we can focus exclusively on generic changes of the intraneuronal endosome transport.

3.4.3 Microtubule-dependent transport of fNDs entrapped in cargos

Microtubules are cytoskeletal scaffolding polymers involved in various processes such as mitosis, maintenance of cell shape, cell polarization and intracellular transport. Microtubule assembly, organization and dynamics in axons and dendrites are still under scrutiny but some fundamental mechanisms are well known (Conde en Cáceres, 2009). For instance, microtubules undergo dynamical instabilities, which consist in cycles of rapid growth (polymerization) and shrinkage (depolymerization). Taxol and nocodazole are two drugs allowing stabilization or destabilization of microtubule network respectively.



Figure 3.12: Nanomolar sensitivity of the fND-tracking assay to nocodazole impairment of the microtubule cytoskeleton integrity. Cumulative probability densities are shown for the control (black), concentrations of 2 nM (green) and 5 nM (red). Inset: bar plots (mean ± s.e.m.). A cartoon illustrate the underlying biological mecanisms. (n>35 trajectories from n=4 embryos for each experiment).

To assess the fNDs-tracking assay sensitivity and to confirm the microtubule-dependent fNDs transport, we perturbed the traffic by destabilizing the microtubule network with nanomolar concentrations

of nocodazole during the course of the 1 h-experiment. It is worth noting that a standard is to use nocodazole between 0.2 μ M to 10 μ M for several hours (Courty et al., 2006; Jaworski et al., 2009). We incubated primary cultures of mouse hippocampal neurons with two concentrations of nocodazole (2 nM and 5 nM), which interfere with microtubule polymerization within few minutes (Vasquez et al., 1997). As shown in Figure 3.12, we observed for 2 nM and 5 nM respectively a decrease in velocity (*p*=3.8x10⁻⁴, *p*=1.4x10⁻⁷ respectively), in run length (*p*=3.4x10⁻⁴, *p*=5.3x10⁻⁶ respectively), in processivity (*p*=4.5x10⁻³, *p*=9.8x10⁻⁵, respectively) and in pausing frequency (*p*=0.54, *p*=0.016 respectively), and an increase in pausing time (*p*=0.003, *p*=6.4x10⁻⁵ respectively). Conversely, perpendicular displacement, longitudinal displacement and the diffusion coefficient were not significantly affected by the addition of nocodazole (*p*-values of *p*=0.989, *p*=0.479, *p*=0.575, respectively, for 2 nM nocodazole, and *p*=0.379, *p*=0.18, *p*=0.544, respectively, for 5 nM nocodazole).

The significant increase in pausing time is as expected for motors unable to overcome the interruption of active transport caused by depolymerization of the plus-end tips of microtubules. This interruption of such an active transport also results in a significant decrease in run length and processivity, and all the intraneuronal transport parameters vary in a dose-dependent manner. Altogether, these results demonstrate the sensitivity of the fNDs-tracking assay as well as the fact that cargos containing fNDs traffic along microtubules, and are therefore fully equipped with kinesin and dynein motors and their specific adaptors.

3.4.4 Dependence of intraneuronal transport on filamentous actin integrity

fNDs-containing cargoes are transported in a microtubule-dependent manner, but it may also involve the actin network. Therefore, we tested the impact on the traffic of cytochalasin D, which is known to interfere with filamentous actin (FA) polymerization (Casella et al., 1981). We incubated primary cultures of mouse hippocampal neurons with either 10 nM or 100 nM concentrations of cytochalasin D (Figure 3.13). Transport parameters were not affected by 10 nM concentration. Conversely, when neurons are treated with 100 nM of cytochalasin D, we observed an increase in processivity and run length (p=0.025, p=0.023 respectively), and velocity, pausing time, pausing frequency, perpendicular displacement, longitudinal displacement and diffusion the coefficient were not significantly affected by the addition of 100 nM cytochalasin D (p= 0.472, p=0.822, p=0.133, p=0.554, p=0.333, p=0.6415) (Figure 3.13).

Using super-resolution microscopy, Xu *et al.*(Xu et al., 2013) showed that there were long FA fibers running along dendritic shafts. Moreover, a dozen of protein families have been identified as candidate cross-linkers between FA and MT (Rodriguez et al., 2003). Therefore, the facilitation of trafficking observed (with 100 nM cytochalasin D) is consistent with co-trafficking involving myosin V/VI and kinesins/dyneins, FA-MT structural interactions, or both. Consequently, a schematic model of intraneuronal endosome trafficking can be proposed (Figure 3.13), in which FA-driven transport opposes the main microtubule-driven motion (left), and/or structural interactions between MT and FA, mediated by cross-linking proteins (in green), affect cargo motion.



Figure 3.13: Nanomolar sensitivity of the fND-tracking assay to cytochalasin D impairment of the actin cytoskeleton integrity. Cumulative probability densities are shown for the control (black), concentrations of 10 nM (green) and 100 nM (red). Inset: bar plots (mean \pm s.e.m.). A cartoon illustrate the possible underlying biological mecanisms. (n>35 trajectories from n=4 embryos for each experiement).

3.4.5 Optically resolving microtubule bundles in living neurons

Unlike axonal microtubule bundles, dendritic microtubule bundles are not aligned (Conde en Cáceres, 2009) as observed by electron microscopy. In live cells, imaging of microtubule was done only in cell lines where microtubule are well separated and not strongly packed and twisted.

As we were interested in imaging the microtubule network in control versus anomalous case, we first started to collaborate with Elisa D'ESTE from Stefan HELL's laboratory at Max Planck for Biophysical Chemistry (Göttingen, Germany). At this time, we thought that STED super-resolution microscopy could resolve microtubule spacing. In this case, both fixation protocol and labeling density have to be optimized to be sure that the structure is well conserved and well sampled, as shown with the discovery of the actin ring in axon using STORM super-resolution microscopy (Xu et al., 2013). Unfortunately, microtubule fine structure was barely resolvable (Figure 3.14a, b). Interestingly, the long-lasting challenge of mapping microtubule bundles

inside primary neurons was recently met using dSTORM super-resolution nanoscopy (Mikhaylova et al., 2015), but yet at young developmental stage (DIV1) in fixed neurons and very close to the soma where microtubule are less packed together and twisted than in distal portion.

Here, the tracking of fND-labeled endosomes can help to solve this problem. Indeed, it is a powerful tool for mapping the intraneuronal microtubule network in living cell, as shown in Figure 3.14c, d. Four trajectories of endosomes driven by kinesin or dynein can be seen in the same dendrite, no more than \approx 400 nm apart (Figure 3.14c). Moreover, adjacent trajectories are separated by a distance of about 40-160 nm which is consistent with reported values (Chen et al., 1992; Mudrakola et al., 2009).



Figure 3.14: Microtubule mapping using the fND-tracking assay. (a-b) Confocal versus STED imaging of microtubule in hippocampal neurons. Immunostaining was done with β 3-Tubulin (a) and MAP2 (b) primary antibody and Alexa561nm as secondary antibody. Scale bar: 1 µm. (c) Trajectories of fND-containing endosomes either kinesin or dynein driven, revealing four underlying well-separated microtubules within the same dendrite. Scale bar: 400 nm. (d) Mapping of microtubule bundles (different cell that in (c)) with single fND-containing endosomes doing back-and-forth motion.

Moreover, considering that microtubules are parallel within a bundle, one can also look for preferential directions in the trajectory of a single vesicle moving back and forth. Practically, straight sections (longer than 3 frames) of the trajectory are linearly fitted (Figure 3.14d). The distribution of trajectory slopes

reveal three main values colorized in green, blue and orange. Because sections with the same slope may belong to the same bundle, we extended the colorized segments on one of their side in order to display what could be a part of the microtubule bundle organization. The three bundles observed appear entangled in the 5-8 μ m *x*-coordinate portion of the trajectory, which is consistent with its local higher level of complexity. Altogether, with additional mathematical processing, the microtubule mapping using the fNDs-tracking assay could be useful for assessing the integrity of the cytoskeleton in both normal and abnormal configuration while performing live cell imaging.

3.4.6 Conclusion 3.4

In this section, we demonstrated that the fNDs-tracking assay is stable and sensitive enough to detect molecular perturbations in the nanomolar range that can impact the intraneuronal transport either directly by depolymerizing microtubule network or indirectly by deconstructing the filamentous actin cytoskeleton. Moreover, this approach can be instrumental to gain insight into the complex intraneuronal transports by selecting either kinesin-driven or dynein-driven transports. Finally, it is a powerful nanoparticule-based methodology to optically resolve microtubule bundles inside freely developing branches of living neurons.

3.5 Conclusion of Chapter 3

In this Chapter 3, we described how we automatically extracted the large number of fND-labelled endosome trajectories using SPT tracking tools. Because the experimental localisation precision knowledge is of utmost importance to draw any robust biological conclusions, we first calibrated our live-cell imaging setup and proved that fNDs coordinates can be determined at a temporal resolution of 50ms with a spatial accuracy better than 30 nm for particles with a signal-to-background ratio larger than 3. Then, we developed a dedicated quantitative trajectory analysis based on the confinement ratio to extract eight trafficking parameters. However, it is worth underlying that a coming standard to infer transient particle transport dynamics in living cells will be to apply Bayesian model selection to hidden Markov modelling, as shown very recently (Monnier et al., 2015).

Thereafter, we proved our approach to be stable during technical and biological duplicates. Moreover, we showed its sensitivity to nanomolar perturbation of either the microtubule or the actin network. Finally, we used the fNDs-tracking assay to gain insight into the complex intraneuronal transports by selecting either kinesin-driven or dynein-driven transports, and to unveil the underlying microtubule network. Altogether, we are now equipped to tackle biological questions related to normal versus dysfunctional cases.

CHAPITER 4

Application to neuropsychiatric-related genetic risk factors

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4.1 Introduction

Neurogenomics is a powerful approach to pre-select a first set of interesting genes and guide neurobiologists to analyse selected pathways. However, the emerging polygenic architecture of mental disorders makes it mandatory to screen the functional impact of genetic variants. Through bioinformatics meta-analysis approach, we have demonstrated the disease relevance of monitoring the intraneuronal transport. We then developed and validated the fNDs-tracking assay which provides spatiotemporal quantification of intraneuronal transport.

This chapter is devoted to the application of the fND tracking assay to measure the functional impact on the intraneuronal transport of brain disease-related genetic risk factors. We first review the neurobiologists ToolBox commonly used for *in vitro* and *in vivo* expression of brain disease-related genes. Then, we evaluate the fNDs-tracking assay sensitivity to subtle changes on the microtubule track (overexpression of Mark1), then on the energy pathway (overexpression of Slc25a12) and eventually on the transcription machinery (haploinsufficiency of Auts2).

4.2 Neurobiologists' toolbox for in vitro and in vivo gene expression

From neurogenomics studies, many genetic variations associated to neuropsychiatric diseases are now available. The effect of these mutations can be mimicked both *in vitro* and *in vivo* by the modulation of gene expression either transiently or constitutively. The former can be performed using transfection (e.g. lipofection, electroporation...) or transduction (e.g. lentivrus, AAV...) for both *in vitro* and *in vivo* studies, whereas the latter can be induced *in vivo* like in transgenic animal models of brain diseases. In this section, we first describe transfection and infection genetic approaches for overproduction or silencing (knock-down) of a protein of interest. Then, we detail the constitutive or conditional knock-in and knock-out of a gene of interest using either homologous recombination or Cre/LoxP system.

4.2.1 Transfection and Transduction

Static or dynamic study of proteins, RNAs or DNA in cultured cells and tissues often use fluorescent probes. In the case of proteins, it is necessary to fuse them with fluorescent proteins as reporters (e.g. GFP, mCherry...). The only way to produce such a chimeric construct inside a cell is to introduce the DNA-coding sequence into the nucleus so that the transcription/translation machinery of the cell itself will produce this exogenous protein. This strategy relies on two critical steps: the DNA-coding sequence production and its introduction into the cell. First, the DNA-sequence coding for the fluorescent fusion protein is genetically engineered from circular bacterial DNA (plasmid) (Figure 4.1a). Then, the plasmid has to be introduced into the host organism to be expressed in the cytoplasm for a short period of time (from few hours to 96 hours after introduction of plasmid DNA into mammalian cells).



Figure 4.1: Lipid-mediated transfection in Mammalian cells. (a) Mechanism of plasmid internalization throughout the plasma membrane mediated by liposomes (From <u>https://www.microscopyu.com</u>). Plasmid circular DNA is shown with the four minimum requirement to perform transfection of exogenous sequence inside cell for fluorescent fusion protein with (1) bacterial replication origin, (2) promoter, (3) cDNA of interest, (4) antibiotic resistance gene. (b) Image of a neuron transfected at DIV2 with the vector TGN38-GFP and imaged with a Leica SP5 confocal microscope at DIV4. Scale bar: 5 μ m.

The plasmid vector used for this purpose has several requisite components. One group of DNA sequence is needed to produce the plasmid itself whereas a second group of DNA sequences will code for the protein of interest. First, the plasmid must contain prokaryotic nucleotide sequences coding for a bacterial replication origin specific to a type of bacteria, usually E. Coli. Moreover, an antibiotic resistance gene (e.g. Ampicillin or Neomycin) or a luminescent protein coding gene (e.g. luciferase) has to be inserted to allow the selection of only the bacteria which incorporated the plasmid. Then, the second group of DNA sequence in the plasmid is related to eukaryotic genetic elements that will trigger the cDNA transcription. A promoter is required to trigger the transcription of the cDNA of interest (Figure 4.1a).

Once the protein-coding plasmid sequence is ready, it has to be delivered throughout the plasma membrane of the cell. A wide spectrum of transfection reagents and techniques are available, ranging from vehicle-mediated delivering (liposome, virus, calcium phosphate precipitation...) to direct internalization (electroporation, microinjection, GeneGun...). The more widespread strategy is the liposome-mediated transfection reagent, which will sequester the plasmid DNA in lipid vesicles that will fuse to the cell membrane and deliver the contents to the cytoplasm (Figure 4.1a). Indeed, the negatively charged DNA will be entrapped inside the positively charges liposomes allowing them to overcome the electrostatic repulsion of the cell membrane. The transfection efficiency is low (around 5% in primary neurons) because either the neurons die before expressing the transgene, or the transgene expression is cytotoxic for the cell or even, the transfected genetic material may never reach the nucleus as it may be disrupted somewhere along the delivery process.

As mentioned in Chapter 2, when we studied the uptake pathway of fNDs inside primary neurons, a colocalization study was performed using transfection of marker of early endosomes, the TGN38 protein. In this experiment, as shown in Figure 4.1b, primary neurons were transfected at DIV2 and imaged at DIV4. The plasmid DNA vector coding for the fusion protein TGN38-GFP and the liposomes (Lipofectamine 2000 reagent, LifeTechnology, ref 11668027) were diluted for 5 min at room temperature in Opti-MEM® I Reduced Serum Medium (Life Technology, ref 31985-062). Then they were mixed together for 20 min at room temperature

with two vortex steps in order to break early liposome complex and increase the probability to get DNA entrapped inside the liposomes. The suspension was diluted in DMEM serum-free medium and incubated for 2 hours. The old complemented neurobasal medium was kept warm and put back. After 24 h to 48 h, neurons express the protein of interest that can be imaged (Figure 4.1c).

Another way to induce exogenous protein expression inside primary neuron is to use viral vectors. The term "**transduction**" is used to describe a virus-mediated DNA transfer into cells. In contrast to transfection of cells with foreign DNA or RNA, viral vectors (retrovirus, lentivirus, AAVs...) are able to infect cells and transport the DNA directly into the nucleus, hence, no transfection reagent is needed. After the release of the DNA into the nucleus, the protein of interest is produced using the host's own machineries. Transduction efficiencies is up to 100% and can be easily achieved without severely affecting cell viability, even in difficult-to-transfect cells, such as primary neurons. Furthermore, some viruses integrate into the cell genome facilitating stable expression. However, transfection is still the method of choice for many applications as construction of a viral vector is a much more laborious process.

Viral vectors can be used either as a transfection substitution for exogenous protein expression in mammalian cell or to regulate endogenous protein expression through siRNA-mediated knock-down (KD). They can be split into four families: retrovirus, lentivirus, adenovirus and adeno-associated virus. Briefly, retroviruses are enveloped particles of about 100 nm in diameter containing two identical single-stranded RNA molecules of 7–10 kb in length. Retroviruses can infect only dividing cells contrary to lentivirus, a subclass of retrovirus, which has the ability to integrate into the genome of non-dividing cells. Then, adenovirus are non-enveloped (without an outer lipid bilayer) particles of about 90–100 nm, containing a double stranded DNA genome. It does not integrate into the genome and is not replicated during cell division. Adenovirus can be used to transduce genes of interest in postmitotic neurons (Lepagnol-Bestel et al. Dyrk1A HMG, 2009).

The last viral vector category is the Adeno-associated virus (AAV). It is a small virus that can infect both dividing and non-dividing cells and may incorporate its genome into that of the host cell but does not replicate. The AAV is non-enveloped virus of about 20 nm in size, with a single-stranded DNA of about 4.7 kb long. The main limitation of AAV is the limited size of DNA that can be integrated (around 2 kb). This viral vector will be used in section 4.4 for siRNA-induce haploinsufficiency of the ASC-related Auts2 gene.

4.2.2 Transgenic mouse model from homologous recombination

Modeling of human brain diseases to understand the function of related genes usually relies on the establishment of transgenic mouse mimicking the effect of genetic mutations (de Angelis et al., 2015; Nestler en Hyman, 2010). As our working hypothesis is to mimic in hippocampal neurons the subtle changes induced by genetic variants, we cannot use the aforementioned transfection strategy that modify gene expression at levels not found in patients with mental disorders. Although transduction can be used for down-regulation of a protein using siRNA, transfection is limited by its low efficiency and uncontrolled protein overproduction.

Therefore, genetically modified mice are required with either constitutive (permanently triggered) or conditional (spatiotemporally triggered) genetic modification leading to endogenous protein expression level. Different type of animals can be used to model neuropsychiatric disorders (zebrafish, *C elegans*, Drosophila...). However, mouse is genetically closest to human and a variety of genetic engineering tools are available for mouse. Furthermore, mouse lines can be obtained in a pure genetic background and phenotypic traits (e.g. memory, social behaviour, stereotypies...) can serve as proxies of abnormal traits found in patients and can be quantified by behavioural studies.

Basically, two strategies have been developed to produce genetically modified mice. The first approach, which is the most common method used to create transgenic mice with specific gene **overexpression**, is based on **random integration** (RI) of exogenous DNA into the mouse genome, by injection of DNA into the mouse pronuclei (this method was instrumental for our study in section 4.3 and 4.4; see Figure 4.4) (Gordon et al., 1980). This procedure involves collecting fertilized eggs at the single cell stage. The two pronuclei containing the genetic material from both the sperm and the egg are visible for a short period of time. At this specific stage, a linearized DNA construct is injected into one of the pronuclei. The injected eggs are then transferred into a foster mice. Generally 10 to 20% of the pups born to the foster mothers have integrated the injected DNA into their genomes, thus becoming transgenic. Yet, as the DNA integrates randomly into the genome, the copy number of the gene of interest cannot be controlled and thus, each pup is a unique founder mouse. Therefore, to check in which part of the mouse genome the exogenous sequence is located and how many copies are integrated, PCR followed by sequencing has to be performed. Moreover, even though a copy was integrated, this does not imply that it will produce a protein (caused by epigenetic, alternative splicing...): a protein quantification is thus mandatory (see for example L38^{het} in section 4.3).

The second approach, pioneered by Mario R. Capecchi, Martin J. Evans, and Oliver Smithies (Nobel Prize in Physiology or Medicine 2007), uses the spontaneous **homologous recombination** (HR) phenomenon that occurs in genome of all species. As this event has a low probability of occurrence in comparison to RI (in the range of 10⁻³), a selection strategy is needed to sort out clones displaying HR in embryonic stem cells (ES cells). Conversely to RI, this strategy is able to generate either **knock-in (KI)** or **knock-out (KO)** transgenic mouse lines (Capecchi, 2005), but require specific design of the exogenous DNA to target a specific endogenous segment.

As shown in Figure 4.2, the experimental procedure is roughly the same for RI and HR, except that for HR, the DNA sequence must be carefully designed. As for transfection, a plasmid is generated with the appropriate DNA sequence of the gene of interest. In parallel, ES cells are extracted from a mouse female embryo displaying totipotent embryonic stem cells at the blastocyst stage and cultivated *in vitro*. The ES cells are then transfected with the plasmid. Here, RI or HR occurs and induces the DNA sequence integration into the genome of the host ES cell. As the phenomenon is very rare, it is necessary to select the few genetically modified ES cells (using antibiotic as these cells also incorporate an antibiotic resistance gene). After purification, the transgenic ES cells are re-injected inside the blastocyst, which are then re-implanted inside a

surrogate mouse. From this step, mosaic animals are mostly generated (i.e. no transgene in the gonads). After the embryo birth and breeding, these animals are subsequently coupled until the transgenic line is stabilized with the exogenous DNA sequence expressed in every cells (Figure 4.2).



Figure 4.2: Induction of specific gene modifications in mice using ES cells. (1) Embryonic stem (ES) cells are cultured from mouse blastocysts, which have the potential to differentiate into any type of cell. The target gene (genetic material to be modified) is identified in the ES cell. (2) Construction of a targeting vector, which contains a cloned gene consisting of regions that are homologous with the target gene and antibiotic and/or antiviral resistance gene to allow ES cell line selection. ES cell transfection (e.g. electroporation, viral infection...). (3) Homologous Recombination, which induces disruption of the target gene. However, random genome integration is also possible and ES cells have to be selected and enriched. (4) Selection and proliferation of taraeted ES cells. followed by (5) microinjection into the blastocyst. (6) The blastocyst was implanted into the mouse, which acts as a surrogate mother and (7) the mosaic embryos are mated with normal mice to generate stable transgenic homozygous. mouse line, appropriate mating will render the mutation (Adapted from http://www.nobelprize.org/).

4.2.3 Cre/loxP recombinase system

The main drawback of the aforementioned HR technique to generate *constitutive* KO transgenic mouse is that it is often lethal for the embryos. However, neurobiologists need to decipher the role of a given gene usually later in development. Therefore, *conditional* transgenesis is required to study the spatio-temporal gene function. As it is often the case in biology, a novel technology was inspired from the Cre–loxP site-specific recombination system of Coliphage, which is a type of *bacteriophage* that infects *Escherichia coli* (Sauer en Henderson, 1988). Cre is a DNA recombinase (38 kDa), which specifically recognizes 34-bp loxP-sites (LOcus of cross-over of P1). Then, Cre–loxP recombination between two sequential loxP sites excises all DNA sequences located between the two sites, forming a covalently closed circle with the ablated gene plus one loxP, and a linear product with the other loxP (e.g. NMDA DNA sequence in Figure 4.3). This Cre–loxP-mediated recombination is highly efficient and precise. In most cases, both loxP sites are placed in the same chromosome and repeated in the same direction (arrow direction in Figure 4.3) so that the desired DNA sequence can be deleted. However, the loxP sites can also be positioned in an opposite direction to create a switch to inactivate and activate genes of interest, or even be placed in different chromosomes for inter-chromosome recombination. In the case of the *AUTS2* project of Prof. SIMONNEAU's team to which I contributed to, the loxP sites have been inserted in two distinct transgenic lines at a distance of 1 Mb.



Figure 4.3: Cre–loxP mediated recombination for regional restriction of gene expression. See main text for details (adapted from (Deng, 2012; Mayford et al., 1996)).

Spatiotemporal gene ablation by Cre recombinase (Figure 4.3) requires two transgenic mouse lines, initially generated by homologous recombination. The first one has to be generated to encompass the

endogenous gene by two loxP sites (NMDA gene in Figure 4.3). The second transgenic mouse is designed so that Cre is express at a given time at a given place in the mouse brain. Usually, a specific promoter is used, such as CamKII, which is expressed specifically in the forebrain CA1 hippocampal region (Figure 4.3). Then, the loxP-modified mice are mated with the Cre-transgenic mouse. This generates a double-transgenic mouse in which the loxP-modified gene has been deleted in a specific cell-type in which the Cre transgene has been expressed (here CA1 pyramidal neurons with KO-NMDA receptor).

Overall, the Cre/LoxP recombinase system is useful to create constitutive or conditional KI or KO in post-mitotic neurons of mouse in a cell type-specific manner. For example, stoichiometric endogenous KI mouse with florescent protein tagging can be performed with Cre/LoxP recombinase system. Also, Cre gene can be expressed directly *in vivo* using recombinant viral vectors or by *in utero* electroporation (IUE). All in all, the Cre/loxP technology has revolutionized the way neurobiologists modulate neural activity *in vivo*. It has made it possible to develop optogenetics with light-activated channels (Madisen et al., 2012) as well as to dissect connections between neuronal networks, which is another field known as connectomics (Livet et al., 2007; Loulier et al., 2014).

4.2.4 Conclusion 4.2

The ability to perform precise genetic manipulations in a cell-type specific manner is essential for analysis of genes, cells, and circuits dysfunction in neuropsychiatric diseases. From coarse gene manipulation with transfection to fine tuning of gene expression with Cre/Lox transgenesis, we showed in this section that numerous neurobiological tools were developed to increase the versatility of applications. Moreover, given the current CRISPR/Cas9 revolution in the field of genome editing (Charpentier en Doudna, 2013), it will become even easier to mimic human brain diseases through a broad spectrum of transgenic animals. Neurobiologists are better equipped to tackle neurogenomics challenges.

4.3 Sensitivity to subtle changes on the microtubule track

In this section, we first review the implication of microtubule-related Mark1 in mental disorders. Then, as we want to mimic the protein over-production level found *post-mortem* in the forebrain of patients with ASC, we detail the establishment of transgenic mouse lines with subtle *Mark1* overexpression. Finally, we evaluate the fNDs-tracking assay sensitivity to subtle changes impacting the microtubule tracking using this transgenic mouse model.

4.3.1 MARK1 is a brain disease-related genetic risk factors

Microtubule-related *MARK1* gene belongs to the microtubule-affinity regulating kinases family, as part of serine/threonine-protein kinases. MARK proteins have the ability to phosphorylate tau protein and related microtubule-associated proteins like MAP2. This mechanism makes it possible to regulate microtubule

dynamics in neurons, and may subsequently regulates dendritic spine morphology and synaptic plasticity (Jaworski et al., 2009). Members of the MARK family were also found to have key roles in cell processes such as determination of polarity, cell-cycle control, intracellular signal transduction, transport and cytoskeleton (Matenia en Mandelkow, 2009). More specifically, *MARK1* phosphorylates MAPT/tau, causing detachment from microtubules (Mandelkow et al., 2004). As hyperphosphorylation of MAPT/tau can lead to its pathological aggregation, this process may be in the early steps of **Alzheimer's disease** (Querfurth en LaFerla, 2010). Interestingly, subtle increase in *Mark1* expression was recently reported in MAPT/tau model of familial Alzheimer's disease, using whole-genome gene expression analysis (Matarin et al., 2015). Furthermore, Pr. Michel SIMONNEAU's group showed in 2008 that several SNVs within the *MARK1* gene were associated with **Autism Spectrum Condition (ASC)**. Moreover, they found *postmortem* that *MARK1* was overexpressed in the forebrain region of ASC patients by about 38% (Figure 4.4a) (Maussion et al., 2008). Finally, we identified the microtubule-associated *MARK4*, a paralog of *MARK1*, being part of the **schizophrenia** NETBAG+ network as SNV *de novo* mutation was discovered on this gene (Fromer et al., 2014)(see Chapter 1 Figure 1.6).

Altogether, these observations highlight the need for studying the functional impact of *MARK1* with an overexpression level close to the one found in patients with mental disorders. As this gene is engaged in the microtubule organization through phosphorylation of MAP2, the monitoring of intraneuronal transport is likely to reveal abnormal endophenotype associated to such a genetic modification.



Figure 4.4: Establishment of transgenic mouse to model Human Brain diseases. (a) MARK1 was found to be overexpressed by about 38% in brain of patients with Autism. From (Maussion et al., 2008) **(b)** SLC25A12 was found to be overexpressed by about 35% in brain of patients with Autism. From (Lepagnol-Bestel et al., 2008) **(c)** Modelling of these subtle overexpressions in transgenic mouse using the Random Integration technique. The plasmid is first generated and then injected inside one pronuclei of a fertilized egg through a micropipette. The egg is inserted back into a female and after 1 years of breeding, the transgenic mouse line is established.

4.3.2 Constitutive overexpression of *Mark1* in the hippocampus

These aforementioned neurogenomics evidence of the implication of *MARK1* in mental disorders prompted us to generate transgenic mice that slightly overexpress *Mark1* genes, in order to mimic the functional impact of genetic variants (Figure 4.4 and 4.5a). Consequently, we used a promoter derived from the *CaMKII*α gene known to give a fine spatio-temporal regulation and to yield slight overexpression at postnatal stages in the neurons of the forebrain of transgenic animals (Mayford et al., 1996).

The generation of this **transgenic construct** consisted in two steps: design of the transgene and injection in C57/Bl6 fertilized eggs. The first step is a transgene plasmid construction following the method described in Section 4.2.1 and performed by Dr. LEPAGNOL-BESTEL in the Pr. SIMONNEAU's group at CPN (protocol detailed in Appendices). Then, the transgene was injected in C57/Bl6 fertilized eggs (CNRS SEAT/TAAM UPS 44, Villejuif, France). It is worth underlying that transgenesis was done in a pure genetic background (C57/B16) that is technically demanding but gives a unique advantage for behavioural tests (many transgenic lines are generated in a mixed genetic background that complicates behavioural test analysis). After stabilization of the construct, two transgenic mouse lines for *Mark1* (namely L38^{het} and L8^{het}) were used for further functional analysis (Figure 4.5).

To quantify the level of *Mark1* gene overexpression in our transgenic mouse, we performed Western blotting (WB) to evaluate the protein level between WT, L38^{het} and L8^{het} (Figure 4.5b, c), and RT-QPCR to evaluate the mRNA level between WT, L8^{het} and L8^{hom} (Figure 4.5d). The first technic was done on hippocampal tissue dissected from P15 mice of *Mark1* transgenic lines. WB was carried out with an antibody against Mark1 and an antibody against actin for normalization. In P15 transgenic mice, we measured 7.4±4.2% (p=0.85) for L38^{het} and 32.0±6.5 % (p=0.011) for L8^{het} overproduction of Mark1 (n=11 WT, n=9 L38^{het} and n=9 L8^{het} respectively). In DIV15 cultured hippocampal neurons, we measured increases of mRNA Mark1 transcript levels of 41.5±4.7% (L8^{het}, p=0.0013) and 87.4±14.9% (L8^{hom}, p=0.0010). RT-qPCR was carried out on the same neurons as were used for the fND-tracking assay (n=3 WT, n=4 L8^{het}, n=3 L8^{hom} respectively). Interestingly, the level of transcript increase is about 41.5% and the level of the protein increase is about 32%, which is consistent with a regulation of the transcript level in cells. Altogether, the quantification of *Mark1* average is about 32%, which is used that we generated a gradient of four Mark1 protein levels, namely WT, L38^{het}, L8^{het} and L8^{hom}, with subtle over-production.

4.3.3 Intraneuronal transport modification correlates with *Mark1*

overexpression.

We then applied the fNDs-tracking assay on these *Mark1* transgenic lines (Figure 4.5e). Quantification of the intraneuronal transport dynamic revealed significant variations for L8^{het} and L8^{hom}, but not for L38^{het} in which no changes were observed in any of the readouts. This is consistent with the absence of *Mark1* overexpression for this transgenic line, therefore acting as another control. In L8^{het} and L8^{hom}, the monotonous

increase in *Mark1* protein levels was accompanied by a monotonous increase in velocity from 1.28±0.04 µm/s (WT) to 1.53±0.09 µm/s (L8^{het}, p=0.03) and 1.64±0.06 µm/s (L8^{hom}, p=0.006), in run length from 0.92±0.07 µm (WT) to 1.22±0.12 µm (L8^{het}, p=0.029) and 1.49±0.13 µm (L8^{hom}, p=0.022), and in processivity from 0.47±0.02 s (WT) to 0.54±0.04 s (L8^{het}, p=0.337) and 0.66±0.08 s (L8^{hom}, p=0.037). We also obtained a monotonous decrease in pausing frequency, from 66.5±2.9 events/min (WT) to 63.8±4.3 events/min (L38^{het}, p=0.787) and 57.6±1.5 events/min (L8^{het}, p=0.044). By contrast, no significant change was noted for pausing time, perpendicular and longitudinal displacements and diffusion coefficient for L38^{het}, L8^{het} and L8^{hom} (p=0.684, p=0.41, p=0.213, p=0.164, respectively, for L38^{het}; p=0.413, p=0.587, p=0.337, respectively, for L8^{het}; p=0.044). However, this quantitative parameter was shown in Chapter 3 to be unreliable, because it yields significant difference in technical and biological replicates.

These results are consistent with a model in which *Mark1* overexpression leads to excess of phosphorylation of the microtubule-associated protein (MAP) and decrease of its binding to microtubule (Mandelkow et al., 2004), thereby acting as a "roadblock" as depicted in the schematic of Figure 4.5f.

Interestingly, when we compare the number of analysed trajectories for n=3 WT versus n=3 L8^{het} (littermates), we are able to record 37 ± 4.2 trajectories/hour versus 55.3 ± 1.5 trajectories/hour respectively (two-sample two-tail t-test p = 0.0142; done in double-blind). This can be interpreted regarding the key role of Mark1 in clathrin-mediated endocytosis, as this protein was shown to interact with the adaptor complex AP-2 of clathrin-coated vesicles (Schmitt-Ulms et al., 2009). Our observations indicate that overexpression of *Mark1* may increase the number of internalization events. Thus, STOP&GO motion of fNDs-labelled endosomes indicative of the clathrin-mediated endocytosis efficiency.



Figure 4.5: Intraneuronal transport modifications are correlated with Mark1 overexpression gradient. (a) Schematic representation of the Mark1 transgene used to create transgenic mouse lines L38 heterozygote (L38^{het}), L8 heterozygote (L8^{het}) and L8 homozygote (L8^{hom}), having different of Mark1 expression levels. (b) Mark1 protein dosage using Western Blot (WB). (c) WB quantification in hippocampus of P15 mice of L38^{het} and L8^{het} transgenic lines (p = 0.85 and p = 0.011 respectively). (d), RT-qPCR quantification of Mark1 mRNA levels in L8^{het} and L8^{hom} (p = 0.0013 and p = 0.0010 respectively) from the same primary hippocampal neuron cultures as the ones used for the intraneuronal transport monitoring shown in (e), after the video acquisitions. Note that quantification of overexpression of Mark1 gene in Mark1 L8^{het} is validated by both molecular biology techniques and that Mark1 L38^{het} acts more like a control experiment. (e) Effects of Mark1 overexpression on intraneuronal transport parameters. We observed an increase in velocity for L8^{het} (p = 0.03) and L8^{hom} (p = 0.006); in run length for L8^{het} (p = 0.029) and L8^{hom}, (p = 0.022) and in processivity for L8^{hom} (p = 0.037). We observe a decrease in pausing frequency for L8^{het} (p = 0.044). We observed an increase in perpendicular displacement for L8^{het} (p = 0.03). 0.044). Yet, this readouts was shown in Chapter 3 to be unreliable, because it yields significant difference in technical and biological replicates. For L38^{het}, the unchanged intraneuronal transport dynamic is consistent with the unchanged Mark1 protein level. **(f)** Mechanistic model of the role of Mark1 protein on intraneuronal transport, as described in (Mandelkow et al., 2004) and consistent with our data. All data are presented as mean \pm s.e.m.; figures written inside the bars represent the number of samples for (c-d) or the number of trajectories/independent cultures for (e). Statistical significance: (*: p < 0.05; **: p < 0.01), see Statistical analysis in Appendices.

4.3.4 Conclusion 4.3

In this section, we reviewed the implication of microtubule-related Mark1 in various brain diseases. We then described the construction and validation of transgenic mouse displaying a subtle overexpression of this gene. When applying the fNDs-tracking assay, we demonstrated that the intraneuronal transport parameters vary in a gene dosage-dependent manner and they change as expected for Mark1-mediated regulation of the microtubule track hindrance. We therefore demonstrated the sensitivity of our assay to detect microtubule roadblock impairments linked to brain diseases, for protein concentration changes as small as $\approx 30\%$.

4.4 Sensitivity to subtle changes of the energy pathway

In this section, we first review the implication of mitochondria-related SLC25A12 in mental disorders. Then, as we want to mimic the protein over-production level found *post-mortem* in the forebrain of patients with ASC, we detail the establishment of transgenic mouse lines with subtle *Slc25a12* overexpression. Finally, we evaluate the fNDs-tracking assay sensitivity to subtle changes impacting the energy pathway using this transgenic mouse model.

4.4.1 SLC25A12 is a psychiatric disease-related genetic risk factor

Mitochondria-related *SLC25A12* is located at 2q24 and belongs to the Ca²⁺-dependent mitochondrial Aspartate-Glutamate Carrier (AGC), encoding the isoform 1 (AGC1). This gene is implicated in oxidative phosphorylation and ATP production (Sakurai et al., 2010; Satrústegui et al., 2007). *SLC25A12* has been associated with different types of **ASC** in various populations, in family-based association studies and case-control studies for Asperger patients (Aoki en Cortese, 2015; Durdiaková et al., 2014; Ramoz et al., 2004). However, these associations were not replicated in all cohorts, highlighting the importance of the individual genetic background (Moore et al., 2013). Importantly, they found *postmortem* that *SLC25A12* was overexpressed in the forebrain region of ASC patients by about 35% (Figure 4.4b) (Lepagnol-Bestel et al., 2008). Finally, we also identified *SLC25A12* as part of the **schizophrenia** NETBAG+ network as SNV *de novo* mutation was discovered on this gene (Fromer et al., 2014) (see Chapter 1, Figure 1.6).

Altogether, these observations highlight the need for studying the functional impact of *SLC25A12* with an overexpression level close to the one found in patients with mental disorders. As this gene is engaged in
the energy pathway of ATP production, intraneuronal transport monitoring can be a relevant proxy to study such an effect.

4.4.2 Constitutive overexpression of SLC25A12 in the forebrain region

These aforementioned neurogenomics evidences of the implication of *SLC25A12* in mental disorders prompted us to generate transgenic mice that slightly overexpress this gene (Figure 4.4c and Figure 4.6a). The *Slc25a12* transgenic line L2^{het} was established following the same procedure as describes in section 4.3.2 for Mark1 transgenic line.

To quantify the level of protein overexpression for *Slc25a12* transgenic mouse, we performed Western blotting (WB) on hippocampal tissue dissected from P15 mice. Because of the choice of a *CaMKII* α promoter, the protein over-production is expected to be limited to the forebrain region only, meaning neocortex and hippocampus. To validate that point, we quantified the level of Slc25a12 in the hippocampus and used the cerebellum as an inner control. WB was carried out with an antibody against Slc25A12 and an antibody against actin for normalization. As shown in Figure 4.5b, compared to WT mice, in L2^{het} mice we observed 32.2±6.9% overproduction of Slc25a12 in the hippocampus (*p*=0.043; *n*=5 WT and *n*=5 L2^{het}), but no variation in the cerebellum (*p*=0.417; *n*=5 WT and *n*=5 L2^{het}). This result is consistent with the role of the *CaMKII* α promoter, validating this construction.

4.4.3 Slc25a12-mediated intraneuronal transport impairment

We then investigated the impact of a small overproduction of Slc25a12 protein on traffic parameters. In P15 transgenic mice, we measured $32.2\pm6.9\%$ (p=0.043) overexpression of Slc25a12 protein levels (Figure 4.6). In cultures of hippocampal neurons (from embryos expressing the same *Slc25a12* transgenic construct) and at DIV 15, we observed decreases in run length from $0.92\pm0.07 \mu$ m (WT) to $0.63\pm0.06 \mu$ m ($L2^{het}$, p=0.022) and in processivity from 0.47 ± 0.02 s (WT) to 0.35 ± 0.03 s ($L2^{het}$, p=0.012). Velocity and pausing frequency were found to be unchanged (p=0.749 and p=0.252 respectively) as well as pausing time, and diffusion coefficient displayed (p=0.092, p=0.873, respectively). However, significant differences in comparison with WT were observed for perpendicular and longitudinal displacements in the case of $L2^{het}$ (p=0.022 and p=0.012 respectively). However, the second parameter was shown in Chapter 3 to be unreliable, because it yielded significant differences in technical and biological replicates. Because both displacements are intrinsically linked in the way we extract them, we decided to consider none of them to interpret the data.



Figure 4.6: Changes of intraneuronal transport dynamic induced by subtle overexpression of Slc25a12. (a) Schematic representation of the Slc25a121 transgene used to create transgenic mouse lines $L2^{het}$. (b) Western Blot quantification of Slc25a12 protein dosage in hippocampus and cerebellum of P15 mice of $L2^{het}$ versus WT (p = 0.043 and p = 0.417 respectively). (c) Effects of Slc25a12 overexpression on intraneuronal transport parameters. We observed an increase in run length (d) (p = 0.022) and in processivity (e) (p = 0.012). Velocity (c) and pausing frequency (f) were unchanged (p = 0.749 and p = 0.252 respectively). Significant differences were observed for perpendicular and longitudinal displacements (p=0.022 and p=0.012 respectively). However, these parameters were shown in Chapter 3 to be unreliable, because they yielded significant differences in technical and biological replicates. Data are presented as mean \pm s.e.m.; means were calculated from the total number of samples (in b) or of independent cultures (in d-g); numbers inside the bars represent number of samples (in b-c) or numbers of trajectories/independent cultures (in d-g). Statistical significance: (*: p < 0.05), see Statistical analysis in Appendices.

4.4.4 Conclusion 4.4

In this section, we reviewed the implication of mitochondria-related *SLC25A12* gene in various brain diseases. We then described the validation of transgenic mouse displaying a subtle overexpression of this gene. When applying the fNDs-tracking assay, we demonstrated the effect of this slight overexpression of *Slc25a12* on the intraneuronal transport. Altogether, these results demonstrate the sensitivity of our tracking

assay to detect indirect intraneuronal transport perturbations, for protein concentration changes as small as \approx 30%. However, although SLC25A12 is required for the mitochondria integrity, its precise molecular mechanism is still unknown. Therefore, additional experiments will be needed to understand how such a slight overexpression can impact the intraneuronal transport. Is it because the ATP production from the mitochondria is disturbed? Is it because the mitochondria dynamic is modified and so it creates obstacles on the microtubule track?

4.5 Sensitivity to subtle changes on the transcription machinery

In this section, we first review the genetic implication of *AUTS2* in neuropsychiatric disorders and its role as a transcription factor. Then, as we want to mimic the haploinsufficiency of this gene (gene expression level decreases by a factor of 2) as observed in patients with ASC and SZ, we detail the down-regulation of this protein using AAV-shAuts2 primary culture of mouse hippocampal neurons. Finally, we evaluate the fNDs-tracking assay sensitivity to subtle changes impacting the Auts2-dependent transcription machinery. All bioinformatics analysis presented in this section were performed by Yann LOE-MIE, PhD.

4.5.1 AUTS2 is a psychiatric-related genetic risk factor

AUTS2 covers more than 1 Mbp locus in the genome and is one of the genes with the largest numbers of rare variants in various neurodevelopmental diseases (Figure 4.7a). Furthermore, *AUTS2* locus displays positive selection between Neanderthals and humans, as expected for a gene involved in cognition (Green et al., 2010). Both duplication and deletion of the whole locus result in abnormal neurodevelopmental phenotypes, indicating the key role of gene dosage as a parameter of phenotypic changes. Furthermore, *de novo* mutations (R152X and A495X; Figure 4.7a) in *AUTS2* locus was recently associated with Schizophrenia (McCarthy et al., 2014). This finding supports the idea of a genetic overlap between ASC and Schizophrenia with a shared genetic etiology.

The *AUTS2* gene encodes a protein located both in the cytoplasm and the nucleus, with a cellular function that remains elusive (Gao et al., 2014; Hori et al., 2014). However, AUTS2 was identified to stimulate the transcription induced by PRC1 (Polycomb Repressive Complex 1), by mediating recruitment of protein kinase CK2 and P300 transcriptional co-activating protein (Figure 4.7b). This result demonstrated, for the first time, the nuclear function of AUTS2 as a transcriptional and chromatin regulator, and its capability to orchestrate specific cellular gene expression profiles (Gao et al., 2014).



Figure 4.7: Human AUTS2 genetic mutation and role as a transcription factor. (a) Schematic representation of the human AUTS2 locus, with variants found in neurodevelopmental diseases. **(b)** A model for the AUTS2-Polycomb Complex-mediated transcriptional activation. Adapted from Gao et al., 2014.

4.5.2 AUTS2-Polycomb Complex regulates intraneuronal transport-related

genes

The aforementioned reports have implicated AUTS2 in neuronal migration and neuritogenesis and in the regulation of expression of a large repertoire of target genes via a Polycomb-dependent remodeling complex (Gao et al., 2014). In this paper, they performed AUTS2 ChIP-seq (Chromatin ImmunoPrecipitationsequencing) for mouse brain and 293T-REx cells (human cells but not from brain). As we were interested in AUTS2 chromatin occupancy in humans and in the brain, we retained only genes present in the intersection of these two gene sets (Figure 4.8a). We then used the Babelomics FATIGO tool (Medina et al., 2010) on the same repertoire (344 genes) to determine the enrichment in gene ontologies (Table 4.1). We found enrichment of this repertoire in the mitochondria and microtubule cytoskeleton Gene Ontology classes ($p_adj=0.0004$ and $p_adj=0.0035$, respectively) (Figure 4.8b and Table 4.1). We finally explored this repertoire with the DAPPLE tool (Rossin et al., 2011), which is a protein-protein interaction network tool (Figure 4.8c). We identified proteins involved in chromatin remodeling (i.e. SMARCA2; SMARCC1) or intraneuronal transport (KIF1A; MAP1B; DNM3, TUBB2B, ACTB, CAPZB). Altogether, these observations prompted us to use the fNDs-tracking assay to check the functional impact of Auts2 haploinsufficiency, and thus validate these neurogenomics findings.



Figure 4.8: Analysis of Auts2-Polycomb target genes. (a) Venn diagram showing the overlap (344 genes) between the target regions of the AUTS2 mouse and human deduced from datasets of (Gao et al., 2014) (b) Enrichment in the mitochondria and microtubule cytoskeleton gene ontology classes (p=0.0004 and p=0.0035, respectively) See Table 4.1. (c) Network of direct protein-protein interactions (PPI) restricted to the 344 genes defined in (a) inferred from DAPPLE analysis. The significance of participation in the PPI network is indicated by a color code (p value of the connections observed versus that expected).

Category	Mitochondria	RiboNucleoProtein Complex	Microtubule Cytoskeleton	Ribosome	Soluble Fraction	Organelle Inner Membrane
Gene Ontology	GO:0044429	GO:0030529	GO:0015630	GO:0005625	GO:0005840	GO:0019866
Fraction in Genome (%)	2.5	2.6	2.3	1.6	1.4	1.4
Fraction in Auts2-regulated Genes (%)	7.6	7.0	6.4	4.9	4.1	4.1
Auts2-regulated Genes	ABCB6, ABCD3, ACSL3, ALAS1, ALDH1B1, DAP3, DNAJC11, GLS, HSPA9, MARS2, MCCC2, MCL1, MRPL32, MRPL41, MRS2, NDUFAF3, NDUFS5, OPA1, PMPCA, RAF1, SDHA, SLC25A25, SLC25A38, TIMM17A, TIMM8A, VDAC1	CDC5L, DAP3, DCP1A, DNM3, FOXO3, HNRNPA0, HNRNPH1, LSM11, MRPL32, MRPL41, NCSTN, NFYA, POP7, PRPF3, RPL10A, RPL22, RPL5, RPL7, RPL7A, RPL7L1, RPS4X, SNRPC, ZNF687, ZNHIT6	ALMS1, APC, CEP120, CEP78, DCTN4, DNM3, FBXO5, FOXO3, HIPK1, HNRNPA0, KIF1A, LYST, MAP1B, MAP4, MAPK14, NFYA, OPA1, PPP2CA, PTP4A1, TBCC, TTK, TUBB2B	ACP1, ACTB, ATG12, COPA, DOCK3, EFNB1, MAP1B, MAPK14, MED22, NAGK, NFYA, PKIA, PPP2CA, PRPS1, SDF4, YWHAQ, YWHAZ	DAP3, DNM3, MRPL32, MRPL41, NCSTN, NFYA, RPL10A, RPL22, RPL5, RPL7, RPL7A, RPL7L1, RPS4X, ZNF687	ABCD3, DNAJC11, MRPL32, MRS2, NDUFAF3, NDUFS5, OPA1, PMPCA, SDHA, SLC25A25, SLC25A38, TIMM17A, TIMM8A, VDAC1
p-value adj.	0.0004	0.0027	0.0033	0.0040	0.0294	0.0384

Table 4.1: Gene ontology (GO) enrichment analysis for AUTS2-regulated genes. p-value adj.: p-value adjusted by Bonferroni correction for multiple comparisons.

4.5.3 Auts2 haploinsufficiency impacts intraneuronal transport

Consequently, we modeled *AUTS2* rare variants by inducing an haploinsufficiency as such variants are generally heretozygous. We silenced *Auts2* in mouse primary hippocampal neurons, with two different AAV constructs expressing shRNAs, to mimic haploinsufficiency of this gene. The two AAVs decreased Auts2 protein levels, as shown on the western blots (Figure 4.9a-c), but only AAV2 did it significantly, by a factor of two (p=0.14 and p=0.025 for AAV1 and AAV2 respectively, compared to control scrambled sh-AAV (SCR); n=5 for each condition). Western blot quantification of *Auts2* haploinsufficiency, with GFP normalization, and the fNDs-tracking assay was conducted in parallel, with the same batch of cells.



Figure 4.9: The experimental haploinsufficiency of Auts2 leads to changes in the intraneuronal transport. (a-c) Experimental haploinsufficiency of Auts2 protein levels (means±s.e.m.; n=5 for each condition) achieved with two Auts2-sh adeno-associated viruses (AAV1 in blue, and AAV2 in orange), compared with scrambled sh-AAV (SCR), for hippocampal neurons 48 hours after AAV infection. (a) shows neurites labelled with GFP to validate the AAV infection. (d) Radar plot showing the effects of AAV2 on trafficking readouts (percentage change relative to the median). (e) Cumulative probability densities with bar plots in the inset (median±s.e.m.) for the eight intraneuronal transport readouts, for control (black), AAV1 (blue) and AAV2 (orange) (n>38 trajectories from n=4 embryos). Statistical significance: (*: p < 0.05), see Statistical analysis in Appendices.

As shown in Figure 4.9d-e, this induced haploinsufficiency was accompanied by a velocity decrease from 1.28±0.07 µm/s (SCR) to 1.11±0.08 µm/s (AAV2, p=0.023), a run length decrease from 0.93±0.11 µm to 0.63±0.18 µm (p=0.020) and a diffusion coefficient decrease from 0.16±0.02 µm²/s to 0.10±0.01 µm²/s (p=0.0064). Moreover, the absence of significant *Auts2* haploinsificiency induced by AAV1 is also consitent with the absence of significant effect on the transport parameters, where the velocity equal 1.24±0.08 µm/s (p=0.88), the run length equals 0.89±0.15 µm (p=0.73) and the diffusion coefficient equals 0.13±0.02 µm²/s (p=0.40). Conversely, for both AAVs, none of the other intraneuronal transport parameters displayed significant changes after 48 h of infection: processivity, pausing time, pausing frequency, perpendicular displacement and longitudinal displacement (p=0.73, p=0.85, p=0.56, p=0.92, p=0.70, respectively, for AAV1; p=0.09, p=0.38, p=0.33, p=0.10, p=0.53, respectively, for AAV2). Altogether, these results demonstrate the functional impact of *Auts2* haploinsufficiency on intraneuronal transport dynamics, which is consistent with the enrichment of Auts2-regulated genes in mitochondria and microtubule cytoskeleton Gene Ontology classes.

4.5.4 Conclusion 4.5

In this section, we first reviewed known relationships between AUTS2 transcription factor and neuropsychiatric disorders. Then, we showed through bioinformatics analysis on recent gene set that among target genes of the AUTS2-Polycomb Complex, those from the GO "Microtubule Cytoskeleton" or from GO "Mitochondria" are significantly enriched, therefore suggesting a deleterious effect on the intraneuronal transport for in ASC patients with *AUTS2* haploinsufficiency. Then, by combining the fNDs-tracking assay and AAV-shRNA-induced *AUTS2* haploinsufficiency, we observed impairment of the intraneuronal transport dynamic, which is consistent with the bioinformatics analysis. Overall, our results demonstrate the sensitivity of our technique to monitor subtle changes in gene expression linked to modifications in the transcription machinery.

4.5 Conclusion of Chapter 4

In this Chapter 4, after a brief review of the neurobiologists' toolbox, we applied the fNDs-tracking assay to study of the functional impact of neuropsychiatric-related genetic risk factors. As a proof-of-principle, we used two common variants (30% overexpression of Mark1 and Slc25a12) and one rare variant (50% down-regulation of Auts2). This leads to three different order of perturbation (1st, 2^{sc} and 3rd respectively) depending on where the perturbation takes place with respect to our system (Figure 4.10). First, we established mouse lines in which microtubule-associated *Mark1* and mitochondria-related *Slc25a12* genes were slightly overexpressed as found *post-mortem* in brains of patients with ASC. Also, we used *in vitro* AAV-shRNA to model of Auts2 haploinsufficiency. Then, we evaluated the fNDs-tracking assay sensitivity to subtle

changes on the microtubule track road (overexpression of *Mark1*), then on the energy pathway (overexpression of *Slc25a12*) and eventually on the transcription machinery (haploinsufficiency of *Auts2*). Whether mutation impact is functionally close to intraneuronal transport mechanism or far from it, we observed significant impairment of the "intraneuronal transport" proxy when mimicking the effect of brain disease-related genetic risk factors. Altogether, we proved that our fluorescent nanodiamond-based methodology is sufficiently sensitive to screen the functional impact of genetic variants, opening the door for future development in translational nanomedicine.



Figure 4.10: Sensitivity of the fND-tracking assay to subtle change in gene expression induced by Autism related-common and rare variants.

CONCLUSION and PERSPECTIVES

Common brain diseases, striking more than 1% of the world population, have a highly polygenic architecture displaying subtle changes in gene expression. Modeling of human brain diseases to understand the function of related genes usually relies on the establishment of transgenic mouse mimicking the effect of genetic mutations (de Angelis et al., 2015; Aoto et al., 2015; Choi et al., 2014). We and others pointed out that intraneuronal transport abnormalities are linked to neuropsychiatric and neurodegenerative diseases (Hirokawa et al., 2010; Millecamps en Julien, 2013). Therefore, we decided to mimic the subtle effect of brain disease-related genetic variants and subsequently quantify their impact on the intraneuronal transport using primary cultures of hippocampal neurons. To this aim, we successfully developed a generic and non-genetic approach to monitor the intraneuronal transport based on tracking of fluorescent nanodiamonds (fNDs) inside branches of living neurons. The high brightness, the perfect photostability and the absence of cytotoxicity make fNDs a tool of choice to perform high throughput long-term bioimaging at high spatiotemporal resolution without any modification of the neuronal homeostasis integrity. We then applied this assay to understand the functional impact of brain disease-related genetic risk factors. We evaluated the technique sensitivity to subtle changes directly impacting the microtubule tracks. In neurons from transgenic mouse, we found that a slight overproduction of the microtubule-related Mark1 protein impacts intraneuronal transport parameters. Using another transgenic line with slight overproduction of the mitochondria-related Slc25a12 protein, we then measured the effect on intraneuronal transport of subtle changes impacting the energy pathway. Finally, we evaluated the assay sensitivity to subtle changes impacting the transcription machinery located in the nucleus by mimicking the haploinsufficiency of Aut2. Whether the changes are functionally close to intraneuronal transport mechanism (e.g. Mark1 and Slc25a12) or far from it (e.g. Auts2), our approach proves to be sufficiently sensitive to detect significant impairment of the "intraneuronal transport" proxy when mimicking the effect of brain disease-related genetic risk factors. To the best of our knowledge, this is the first direct measurement of the functional impact of brain disease-related genetic risk factors using a nanoparticle-based technology.

As a **horizontal development**, using the CRISPR/Cas9 genome editing technology(Doudna en Charpentier, 2014), it is possible to model multiple brain disease-related genetic risk factors in transgenic animals. Our fND tracking methodology is adapted to understand how these multiple genetic variants interact with each other to impair the intraneuronal transport (Figure 5.1a). Another approach would be to directly tackle the full polygenic complexity of each disease. The use of human induced pluripotent stem cells (hiPSC) from patients with brain disease is becoming a standard to address medicine questions (Dolmetsch en Geschwind, 2011; Paşca et al., 2014; Wang et al., 2014). Starting from skin biopsy cells, hiPSC could be differentiated into neurons and once a mature neuronal network is established, the fNDs-tracking assay could be applied (Figure 5.1b). With automation of our fNDs-tracking assay to perform high-throughput high-

content screening, this approach can be instrumental to develop an unbiased diagnosis of neuropsychiatric and neurodegenerative diseases, paving the way for future development in translational nanomedicine.



Figure 5.1: (*a*-*b*) Horizontal developments either for rare de novo variants screening or for translational application. (*a*) Neuron culture from CRISPR/Cas9-mediated transgenic mouse to mimic rare de novo variants. From (Charpentier en Doudna, 2013). (*b*) hIPSC-derived neuron cultures to mimic polygenic complexity of each disease. Adapted from (Urban en Purmann, 2015). (*c*-*d*) Vertical developments on either the nanotool or the optical system to improve the versatility of our fNDs-tracking assay. (*c*) Covalent functionalization of fNDs with anti-GFP nanobody. Adapted from (Triller en Choquet, 2008). (*d*) Lifetime imaging of fNDs deep in brain slices Time-gated microscopy setup to image fNDs in brain slices with FirstLight OCAM²S EMCCD incorporating an integrated electronic shutter. EMCCD clock is used as master for the synchronization. Inset: schematics of fluorescence decay for tissue autofluorescence (dashed line) versus fND fluorescence (plain line), displaying also the gate of delayed detection. DDG: digital delay generator adjusting the delay between the laser pulse and the camera shutter; L: lens; BE: beam expander; M: mirror; DM: dichroïc beamsplitter; Obj: high numerical aperture microscope objective; BFP: objective back focal plane.

As a **vertical development**, it should be interesting to improve the protein specificity of fNDs through covalent functionalization with antibody, nanobody and intrabody (antibody that works within the cell to bind to an intracellular protein (Fukata et al., 2013)). Then, after receptor-mediated internalization or microinjection, monitoring of their intraneuronal transport of the protein of interest could be performed (e.g.

synaptic receptor screening from endocytosis/exocytosis trafficking to long-range transport). The unique photophysical properties of fNDs should provide invaluable insight on diverse neurobiological mechanisms such as the dynamic of receptor-specific endosomes or the cooperation of adjacent synapses (Figure 5.1c).

In a close future, another vertical development will concern the optical system itself, to extend our fNDs-tracking assay from monolayer primary culture to 3D-organotypic culture and brain slices. This will be the first steps to the very challenging goal of analyzing intraneuronal transport between two brain sub-regions in freely moving mice. Because of their thickness (few tens of μ m), brain slices or organotypic cultures exhibit some autofluorescence which reduces the nanoparticle fluorescence signal-to-background ratio (SBR), and makes the tracking of the fNDs-containing cargos harder. In order to maintain a high SBR, we will take advantage of the long radiative lifetime of fNDs (≈ 25 ns against ≈ 2 ns for autofluorescence) and make a temporal selection of the photons of interest. To this aim, we will develop a wide field time-gated imaging setup with 95% detection efficiency, high-speed acquisition (up to 2067 frames/s) and MHz pulsed laser repetition frequency (Fig. 5.1). We will use an EMCCD with a built-in fast electronic shutter (200 ns closing duration) developed by the spinoff FirstLight (http://www.first-light.fr/category/ranges/shuttered-emccd/). The ultrafast acquisition rate can also be used to provide images in single photon counting regime, opening the possibility of a second filtering step. The combination of these two levels of "filtering" should improve the SBR from fNDs, thus making it possible the tracking fNDs despite the surrounding autofluorescence from thick biological tissue (Figure 5.1d).

The long-term perspectives of this project is to monitor the intraneuronal transport using non-genetic tagging directly in freely behaving mice (control versus transgenic). This requires imaging with distal optical element inserted deep inside the brain. Such miniaturized endoscopic system have been developed over the past decade by the team of Mark Schnitzer at Stanford University (Barretto et al., 2009; Jung en Schnitzer, 2003), who built his instrument relying on index gradient lenses optimized for multiphoton microscopy. This approach was recently successfully applied to study subgroups of neurons, namely island and ocean neurons of the entorhinal cortex, known to be involved in specific type of learning and memory (Kitamura et al., 2015; Sun et al., 2015).

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A.1 Materials and Methods

All experiments were approved by the Institut National de la Santé et de la Recherche Médicale (INSERM) animal care and use agreement (D-13-055-19) and the European community council directive (2010/63/UE).

A.1.1 Primary hippocampal neuron cultures

For wild type neuron cultures, hippocampi from E18.5 OF1 mouse embryos were isolated in a dissecting medium (HBSS-Hepes) and at least four hippocampi were pooled. Hippocampal neurons were then enzymatically dissociated (0.25% trypsin), mechanically triturated and plated on LabTek II (Nunc, USA) dishes (2x10⁵ cells per well) coated with poly-DL-ornithin (Sigma-Aldrich, USA), in DMEM (Invitrogen, USA) supplemented with 10% foetal bovin serum (FBS), 0.1% GlutaMAX (Invitrogen) and 1% PenStrep (Invitrogen). Two hours after plating, DMEM was replaced by Neurobasal phenol-free medium (Invitrogen) supplemented with 0.1% GlutaMAX (Invitrogen), 2% B27 (Invitrogen) and 1% PenStrep (Invitrogen). Cultures from C57Bl6 transgenic mouse were conducted separately for each embryo and genotyping was performed elsewhere.

A.1.2 fNDs internalization

Hippocampal neuron cultures from Day In Vitro (DIV) 15 to 16 were used unless otherwise stated. The maintaining medium was set aside and replaced by 0.5 ml of Neurobasal phenol-free medium (Invitrogen) to which we added 5 μ l of \approx 28 nm-sized fND stock solution (0.5 mg/ml). After a 10 min incubation duration, the medium was replaced by the old maintaining medium set aside. LabTek was then placed in the incubator during 20 min before starting the video acquisition.

A.1.3 Cytoskeleton destabilizing-drug experiments

Nocodazole and Cytochalasin D powder were purchased from Sigma-Aldrich (St Louis, MO, USA). Following supplier recommendation, Nocodazole and Cytochalasin D were kept in stock solutions of 5 mg/ml and 1 mg/ml respectively in dimethylsulfoxide (DMSO) at temperature of -20°C. Nocodazole (Cytochalasin D respectively) was added to the cultures after fND protocol internalization at final concentrations of 2 nM and 5 nM (10 nM and 100 nM respectively. The DMSO volume added to each well was adjusted to be 0.5% of the total medium volume per well.

A.1.4 *Mark1* and *Slc25a12* DNA extraction and genotyping

DNA extraction: Tissue from mouse hippocampus embryo was digested overnight at 55°C in the digesting buffer: Tris HCl pH=8 (50 mM), EDTA pH=8 (100 mM), NaCl (60 mM), SDS (1% w/v), complemented with 15 μ l of proteinase K (Qiagen N.V., Netherlands). Inactivation was done at 99°C for 10 min and 3 μ l of 1% (w/v) RNaseA (Thermo Fisher Scientific, USA) was added prior to incubation at 37°C for 90 min. DNA was then extracted in 300 μ l of Phenol/Chloroform, and after 5 min centrifugation at 13200 rpm, aqueous phase was diluted elsewhere with 10% of sodium acetate (3 M concentration). Then, 800 μ l of cold ethanol were added before freezing the solution at -80°C for 30 min, followed by 30 min of 13200 rpm centrifugation at 4°C.

Supernatant was removed and the pellet was rinsed at least once with ethanol 70%. The pellet was finally dried at room temperature and resuspended in sterile water.

Genotyping (performed by Christine PLANCON): PCR was then performed using primers for *Mark1* and *Slc25a12* as follow: UPCamKII[®] GACTAAGTTGTTCGCATCC; DW_Mark1: TGACGTAATGGAGTTTCTACAC; DW_Slc25a12: TTTCAAATGTCACCTCTCCA. Each PCR amplification was prepared by mixing 0.5 U Hifi taq DNA polymerase (Life Technologies, USA) 1 µL of 10x Buffer, 2 mM MgSO₄, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1 µM of each of the corresponding primer, and 50 ng of genomic DNA A final volume of 10 µL was achieved using water. A negative control using all reagents except template DNA was also included. Reactions were carried out in a Mastercyler[®] pro S thermocycler (Eppendorf, Germany) using the following cycling conditions: 2 min at 95°C followed by 28 cycles of 95°C for 15 sec, 58°C (for *Mark1*) or 56°C (for *Slc25a12*) for 30 s, and 72°C for 30 s. A final extension step at 72°C for 7 min was performed after the cycles. After this step, samples were kept at 4°C until being electrophoresed in 1.5% agarose 1xTBE gels. For visualization of electrophoresed PCR products, gels were stained with 1xSYBR[®] Safe DNA Gel Stain (Life Technologies) and digital images were captured in a InGenius 3 (Syngene, UK).

RNA extractions were performed either by me or by Julia VIARD, PhD student and *RT-QPCR* were performed either by Christine PLANCON or by Aude-Marie LEPAGNOL-BESTEL, PhD.

A.1.5 Protein extraction and Western Blot

Hippocampus were dissected from P15 Mark1 and Slc25a12 transgenic mice and proteins were extracted in 100 µl of lysis buffer: Tris HCl pH7.4 (20 mM), NaCl (100 mM), NP40 (1% v/v) and protease phosphatase inhibitor dose (Thermo Fisher Scientific). After milling and restoring for 30 min on ice, centrifugation was performed at 13200 rpm at 4°C for 10 min. Supernatant was then removed and kept at -80°C until Western Blots were performed. Protein electrophoresis migration was performed in 4-20% Mini-PROTEAN Tetra TGX gels (Bio-Rad, USA) in a TGS buffer (Tris, 5 mM; Glycine, 192 mM; 0.1% SDS, pH 8.3) with 40 µg of protein lysate after denaturation for 10 min at 95°C. Nitrocellulose membranes (Trans-Blot Turbo, Bio-Rad) were used for protein transfer. Membrane was then blocked in TBS 1X Tween 0.1%, 5% non-fat milk for 1 hour at RT under shaking. Primary antibody Mark1 (ref. 3319S, Cell Signaling, USA) and Slc25a12 (ref. ab107436, Abcam, UK) and Auts2 (ref. ab96326, Abcam, UK) were incubated overnight at 4°C either in 5% BSA and in 5% milk respectively. Normalization was performed using Actin (ref A3854, Sigma Aldrich, USA) or GFP (ref A290, Abcam, UK) staining. Secondary HRP-conjugated antibodies were used in corresponding species and enzyme reaction was activated using Clarity Western ECL Substrate (GE Healthcare, USA), and imaged with ChemiDoc XRS (Bio-Rad). For Auts2 experiment, proteins were extracted 48 h after AAVScramble, AAV1 and AAV2 infection, and five 100 mm-petri dishes at a density of 2.10⁶ neurons were infected for each condition. Slc25a12 and Mark1 quantification normalized to Actin were performed using images acquired at the best dynamic range (very few saturated pixels).

A.1.6 Statistical analysis

Comparison between two data sets was performed with the statistical analysis tools integrated in Igor Pro (WaveMetrics, USA). The pipeline analysis first applies a Kolmogorov-Smirnoff rank test of the null hypothesis "data follow a Gaussian (normal) distribution". In most cases, normality hypothesis was rejected and thus a Mann-Whitney-Wilcoxon comparison two-tailed test was performed. When the normality hypothesis could not be rejected, two-sample two-tailed t-test was performed, after evaluating the null hypothesis of equal variance. Stars are referred to the following significance level: * for p<0.05; ** for 0.001 ; *** for <math>p<0.001. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. Data collection for transgenic neurons was performed blind to the conditions of the experiments.

A.1.7 Preparation of Hippocampal Slices

Dirk1A 189N3 KI and WT littermate male mice aged 17–22 week-old were shipped from IGBMC Strasbourg to INMED Marseille. Mice were deeply anesthetized with xylazine 13 mg/kg / ketamine 66 mg/kg and transcardially perfused with a modified artificial cerebrospinal fluid (mACSF) containing the following (in mM): 132 choline, 2.5 KCl, 1.25 NaH2PO4, 25 NaHCO3, 7 MgCl2, 0.5 CaCl2, and 8 D-glucose prior to decapitation. The brain was then removed rapidly, the hippocampi were dissected, and transverse 450 μM thick slices were cut using a Leica VT1200S vibratome in ice-cold oxygenated (95% O2 and 5% CO2) mACSF. Slices recovered at room temperature for at least 1 h in artificial cerebrospinal fluid (ACSF) containing the following (in mM): 126 NaCl, 3.5 KCl, 1.25 NaH2PO4, 26 NaHCO3, 1.3 MgCl2, 2.0 CaCl2, and 10 D-glucose. Both cutting solution and ACSF were between 290mOsm and 310mOsm. All solutions were equilibrated with 95% O2 and 5% CO2, pH 7.4.

A.1.8 fEPSP Recordings

Acute slices were individually transferred to a recording chamber maintained at $30-32^{\circ}$ C and continuously perfused (2 ml/min) with oxygenated ACSF. Field excitatory postsynaptic potential (fEPSP) were made in the lucidum of CA3 area with glass electrodes (2–3 MΩ; filled with normal ACSF) using a DAM-80 amplifier (low filter, 1 Hz; highpass filter, 3 KHz; World Precision Instruments, Sarasota, FL). Mossy fiber-mediated fEPSP were evoked by weak electrical stimulations performed via a bipolar NiCh electrode (NI-0.7F, Phymep, Paris) positioned in the lucidum of CA3 area; the stimulus intensity, pulse duration, and frequency were around 30V, 25µs, and 0.1 Hz, respectively. Data were digitized with a Digidata 1440A (Molecular Devices) to a PC, and acquired using Clampex 10.1 software (PClamp, Molecular Devices). Signals were analysed off-line using Clampfit 10.1 (Molecular Devices). LTP was induced by tetanus at 25Hz during 5s; D-APV (D-2-amino-5-phosphonovalerate, 40 µM) was included in ACSF during the tetanus to eliminate contamination of MF-CA3 LTP with NMDA receptor-dependent component. At the end of each experiment,

2μM DCG-IV, a group II mGluR agonist, was bath applied to confirm the mossy fiber synaptic origin of fEPSP recorded in CA3. The magnitude of long-term plasticity was determined by comparing baseline-averaged responses before induction with the last 10 min of the experiment. Example traces are averages of at least 30 consecutive sweeps taken from a single representative experiment.

For all reagents, stock solutions were prepared in water or DMSO, depending on the manufacturers' recommendation, and stored at -20°C. Upon experimentation, reagents were bath applied following dilution into ACSF (1/1000). D-APV and DCGIV were purchased from Tocris Bioscience. Salts for making cutting solution and ACSF were purchased from Sigma.

A.1.9 Sample Preparation for dSTORM nanoscopy

Cell cultures were fixed for 20 min in PBS1x, pH 7.4, containing 4% paraformaldehyde (PFA) and 1% sucrose, followed by three washing steps. Fiducial markers (TetraSpeck microspheres, 100 nm diameter, Invitrogen T7279) were attached to the coverslips after fixation (1:200 dilution) for 5min at room temperature (RT). Then, fixed neurons were permeabilized with 0.3% Triton X-100 for 10 min at RT, then blocked with PSB1x containing 0.1% Triton X-100 and 3% BSA, and then labelled over night at 4° with antibodies against Auts2 (Atlas antibodies, HPA000390, 1:200) and SYNPO (Synaptic Systems, 124 011, 1:500) followed by Alexa Fluor 647 (Invitrogen, 1:250–500) and AntiGuineaPig-Cy3 (Jackson Antibody; 1:500 dilution) secondary antibodies (1h at RT). Finally, Phalloïdine-Alexa488 (LifeTech, 1 μ /cvs) was applied for 10min at RT for actin staining. dSTORM was conducted in PBS (pH 7.4), containing 10% glucose, 50 mM b-mercaptoethylamine, 0.5 mg/ml glucose oxidase, and 40 mg/ml catalase, degassed with N2 (Specht et al., 2013).

A.1.10 dSTORM super-resolution imaging

Single-molecule imaging was carried out as described elsewhere (Izeddin et al., 2011) on an inverted Nikon Eclipse Ti microscope with a x100 oil-immersion objective (N.A. 1.49), an additional x1.5 lens, and an Andor iXon EMCCD camera (image pixel size, 107 nm), using specific lasers STORM of Alexa Fluor 647 (405 and 639 nm). Movies of 2.104 frames were acquired at frame rates of 50 ms with EMgain of 300. The z position was maintained during acquisition by a Nikon perfect focus system. Conventional fluorescence imaging was conducted with a mercury lamp and specific filter sets for the detection of Alexa 488 (excitation 485/20 nm, emission 525/30 nm) and Cy3 (excitation 560/25, emission 607/36).

A.1.11 Super-resolution image reconstruction

Super-resolution image reconstruction was carried out using the software ThunderStorm (Ovesný et al., 2014; Sage et al., 2015). First, cross-correlation was used for drift correction. Then, data point with less than 500 photons and with a localisation precision higher than 20 nm were filtered out and finally, points with

less than 5 neighbours within an area of 30 nm were also filtered out. Data rendering was performed using a 2D-gaussian of standard deviation equal to the localisation precision, with a final pixel size of 10 nm. Once Auts2 dSTORM super-resolution image was merged with actin conventional image, dendritic spines were manually selected. The number of Auts2 clusters per sub-region were counted and using the ImageJ plugin, we measured the spine area, and the spine length and head width (minimum and maximum Feret diameters).

A.2 NetBag+ Network



Figure A.1: Complete NETBAG+ network of Figure 1.6 including all GO classes.

A.3 Photophysical properties of fluorescent probes

Atoms and molecules present a proper electronic structure associated to a well-defined electronic states and energy values. Excited states can be populated by either a physical (e.g. absorption of light), mechanical (e.g. friction), or chemical mechanism (Figure A.2). When this atom or molecule goes back to its ground state, a photon is emitted and thus fluorescence lights up. The term photoluminescence refers to the generation of a photon due to photon excitation whereas cathodoluminescence refers to the generation of a photon due to photon excitation whereas cathodoluminescence refers to the generation of a photon due to electron excitation. Photoluminescence occurring within few pico to nano-seconds after light excitation is named fluorescence while it is named phosphorescence when it occurs on few milliseconds to seconds timescales. Overall, fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength, and re-emit it at a longer wavelength (Lakowicz, 2006).

Figure 2.2 displays the Jablonski diagram accounting for the absorption and emission phenomena of a photoluminescent organic molecule with spin singlet symmetry in the ground (S_0) and excited states (S_n , $n \ge 1$) and triplet symmetry in metastable lower energy state (T_1). Briefly, to excite a molecule from the ground state S_0 to the first excited state S_1 , a photon has to be absorbed and its energy has to be at least equal to the energy difference between the lower vibrational energy level of S_1 state and the lower vibrational energy level of S_0 state. After vibrational relaxation (few picoseconds), the de-excitation of the system happens with the emission of a photon having an energy (respectively a wavelength) lower (respectively higher) than the incoming one. The energy difference corresponds to the so-called Stokes shift. If the de-excitation of the emitter happens from the metastable state, we obtain phosphorescence.



ground state

Figure A.2: Simplified Jablonski diagram displaying energy levels of an organic dye. After an electron absorbs a photon the system is excited electronically and vibrationally. The system relaxes vibrationally, and eventually fluoresces at a wavelength longer than the excitation laser one.

Fluorescent probes can be characterized by a set of physical properties that are important to know for biologist in order to optimize experimental protocols:

Absorption, emission and excitation spectra: they are the "fingerprints" of each fluorophore. The absorption spectrum is the continuous spectrum or band pattern that appears when electromagnetic radiation (i.e. light) is absorbed by the fluorophore over a spanning frequency range of the excitation source. The emission spectrum is the continuous spectrum or bright band pattern formed when light is emitted by the fluorophore for a given excitation wavelength. The excitation spectrum is obtained by monitoring the fluorescence intensity at a given wavelength (usually the one of the maximum intensity of the fluorophore) while the fluorophore is excited over a spanning frequency range.

Quantum Yield: it is the relative efficiency of the fluorescence (described by the radiative decay rate k_r) compared to other de-excitation processes (described by the non-radiative decay rate k_{nr}):

$$\eta = \frac{k_r}{k_r + k_{nr}} \qquad (2.1)$$

It can also be seen as the ratio of the emitted to the absorbed photons. Its maximal value can be 1 in absence of non-radiative de-excitation processes.

Extinction coefficient and absorption cross-section: the extinction coefficient (or molar absorption coefficient, ε) measures the strength of a molecule to attenuates light at a given wavelength. For light propagating through a solution of a fluorophore M, its intensity at a distance *L* is described by the Beer-Lambert law: $I(L) = I(0) \varepsilon [M].L$, with I(0) the incident intensity, [M] the molar concentration of the fluorophore M in solution. Thus the extinction coefficient ε is the quantity of light absorbed for a specific wavelength and is measured in (mol.L⁻¹)⁻¹.cm⁻¹. The molar absorption coefficient is related to the **absorption cross-section** σ (usual unit is cm²) via the Avogadro constant as: $\sigma = 3.82 \times 10^{-21} \varepsilon$.

Fluorescence decay lifetime: it is the characteristic duration that a fluorophore remains in its excited state after excitation, and is defined as:

$$\tau = \frac{1}{k_r + k_{nr}} = \frac{\eta}{k_r} \qquad (2.2)$$

In the case of organic dye the fluorescence lifetime usually depends on the physico-chemical environment properties, like pH, solvent dipole orientation, ionic force...

Photostability: it characterizes the stability of the fluorescence signal under continuous excitation. Photobleaching of a fluorophore refers to the permanent loss of their fluorescence property after some time of illumination. The reasons of photobleaching vary and are not fully understood but the commonly accepted explanation is the reaction of the fluorophore in its triplet state with oxygen molecules leading to the formation of a new, non-fluorescent, molecule. Photoblinking is another phenomenon related to photostability, in which the fluorescence is interrupted by dark periods.

Altogether, an ideal fluorescent probe for long term bioimaging should have well separated absorption and emission spectra, a not too broad emission spectrum (to avoid overlap with other emitter), a quantum efficiency close to 1, a large extinction coefficient (to avoid high excitation powers), a perfect photostability and low cytotoxicity. Moreover, as shown in Figure 2.1b, excitation and detection in the far red and near infrared (NIR) region are ideal for *in vivo* imaging since biological tissues are more transparent to NIR photons (Guo et al., 2014; Lukinavičius et al., 2013).

A.4 Articles

A.4.1 "Fluorescent nanodiamond tracking reveals intraneuronal transport abnormalities induced by brain disease-related genetic risk factors", *Nature Nanotechnology (waiting for revision)*

A.4.2 "Chromosome 21 large-scale yeast two hybrid screening uncovers chromatin and synaptic complexes involved in intellectual disabilities and related to Late Onset Alzheimer's disease", *PNAS* (submitted)

A.4.3 "AUTS2, a gene implicated in psychiatric and neurodevelopmental disorders is a modulator of AKT2 in dendritic spines", Science (in preparation)

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Fluorescent nanodiamond tracking reveals intraneuronal transport abnormalities induced by brain disease-related genetic risk factors

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Common brain diseases, such as autism and Alzheimer's disease, each striking more than one percent of the world population, have a highly polygenic architecture displaying subtle changes in gene expression¹. Abnormalities of the intraneuronal transport have been linked to genetic risk factors found in patients^{2,3}, pointing out the relevance of monitoring this key biological process. However, current methods to measure the intraneuronal transport have limitations that prevent the detection of its slight modifications. Here, we report a sensitive approach based on tracking of fluorescent nanodiamonds (fNDs) inside branches of dissociated neurons. The high brightness, perfect photostability and absence of cytotoxicity⁴ make fND a probe of choice to perform high-throughput long-term tracking in living neurons with 12 nm spatial and 50 ms time resolutions. As a proof-of principle, we applied the fND-tracking assay on two transgenic mouse lines mimicking the slight protein concentration changes (≈30%) found in brain of patients. In both cases, this nanoparticle-based methodology proves sufficiently sensitive to detect these subtle changes, paving the way for developments in translational nanomedicine.

A polygenic architecture of brain diseases as diverse as autism spectrum disorders, schizophrenia and late-onset Alzheimer's disease is emerging, with the discovery of interplay between hundreds of genetic variants and subtle changes in gene expression, later referred as genetic risk factors¹. The modeling of human brain diseases to understand the function of related genes usually relies on the establishment of transgenic mouse mimicking the effects of genetic changes⁵. As intraneuronal transport abnormalities have long been recognized to be linked to neuropsychiatric and neurodegenerative diseases^{2,3}, we decided to mimic *in vivo* the subtle changes induced by brain disease-related genetic variants and subsequently quantify their impact on the intraneuronal transport using primary cultures of mouse hippocampal neurons.

Previous attempts to quantify intraneuronal transport have been based on cargo labelling with genetic constructs of chimeric fluorescent proteins^{6,7}. Limitations of this strategy are low transfection yield, uncontrolled protein overproduction, photobleaching of the reporters, and substantial cytotoxicity. Fluorescent semiconductor nanocrystals (quantum dots, QDs) have been widely used to probe a variety of cellular events⁸, owing to their high photoluminescence yield and good photostability. However, attempts to use QDs to study intraneuronal transport have

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been limited to the specific model of long axons of dorsal root neurons cultured in microfluidic devices^{9,10}. Moreover, the toxicity of QDs constitutive elements potentially limits their use in long-lasting studies¹¹ and QDs blinking impedes high spatiotemporal resolution tracking. Considering these intrinsic limitations, there is a need for developing new generic approaches able to screen the functional impact of brain disease-related genetic modifications.

Here, we developed a novel quantitative assay based on tracking of fluorescent diamond nanocrystals (fluorescent NanoDiamond, fND) in mouse hippocampal neurons (Fig. 1, Supplementary Videos 1 and 2). We used 30 nm-sized fNDs (Supplementary methods and Fig. S1a), whose fluorescence stems from nitrogen-vacancy (NV) color centers created within the nanocrystals and acting as perfectly photostable and non-blinking emitters in the far-red spectral domain¹² (Supplementary Fig. 1b). Such remarkable properties have been already exploited in bio-applications requiring long-term tracking^{13,14}. Moreover, fNDs have been reported to show a notably low cellular toxicity in primary neurons¹⁵.

Fig. 1a shows the different steps of the fND-tracking assay, from neuron dissociation to video acquisition. To introduce fND into neurons, we used the endocytosis mechanism¹⁶. We subsequently tracked fND-containing endosomes by pseudo-Total Internal Reflection Fluorescence (pseudo-TIRF) video microscopy, with high-throughput (Fig. 1b) and 20 Hz frame rate, and then made a thorough quantitative analysis of their motion. Pseudo-TIRF microscopy excites only the region of interest, a few micrometers in depth above the culture glass substrate (Supplementary Fig. 1c), leading to a high signal-to-background ratio (SBR). We determined the localization precision of the setup for different SBR, using fNDs immobilized on a coverglass (see Methods), and achieved a localization precision smaller than 30 nm for SBR>3 (Fig. 1c). For the quantification of intraneuronal transport parameters, trajectories with more than 30% of constitutive points having a SBR<3 were excluded. Using such a filter, we obtained a mean localization precision of 12 nm over all trajectories (Inset of Fig. 1c).

Fig. 1d displays a white-light illumination image of a main neuronal branch and its smaller branches, merged with one frame of Supplementary Video 1 showing fNDs at different locations (red spots), two of which moving in the same branch (Fig.1e). Fig. 1f shows the kymograph of these two fNDs, highlighting the "stop-and-go" behavior characteristic of organelle transport in neurons¹⁷. For precise quantification of this

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motor-driven activity, we first extracted the fNDs trajectories from the videos and identified the "stop" and "go" phases (Supplementary Fig. 2, Fig. 1g) from which we inferred six intraneuronal transport readouts: velocity (in μ m/s), run length (in μ m), processivity (in seconds), pausing time (in seconds), pausing frequency (events per minute) and the diffusion coefficient (in μ m²/s).



Figure 1 | Intraneuronal transport monitoring by fluorescent nanodiamond tracking. a, Experimental pipeline from hippocampal neuron culture dissociated from E18.5 mouse embryo to endosome trajectory acquisition using pseudo-TIRF microscopy. DIV: day in vitro. b, Histogram of dataset sizes, i.e. number of trajectories exploited for readouts guantification. The mean value is 37 trajectories/run (n=56 runs). c, Localization precision versus signal-to-background ratio (SBR) of fNDs immobilized on a glass coverslip (from ~100 fNDs imaged over 200 frames; data plotted as mean+/standard deviation). By applying a cut-off at SBR=3, we obtained a localization precision better than 30 nm. Inset: distribution of the mean localization precision for each fND-containing endosome trajectory (n=460 trajectories from 11 wild type experiments). **d**, Transmission white-light illumination image of the neuronal branches merged with the fluorescence channel (extracted from Supplementary Video 1) showing four fNDs moving within dendrites (yellow arrows). The cell soma associated with the main branch is on the left (not visible). During the two-minute movie, two fND-containing endosomes, labeled #1 and #2 (solid white line box) were observed moving towards the cell soma, in the same branch. e, Superimposition onto a white light image of the positions of these two fNDs (#1, in yellow; #2 in green), determined by particle tracking, with a persistence of 10 s, at different times. Scale bars: 5 μ m in (d) and 1 μ m in (e). f, Kymograph of the motion of fNDs #1 and #2 shown in (e)(white box), illustrating their "stop-and-go" behavior. Scale bars: 15 μ m (space) and 10 s (time). g, Top: trajectories of fND #1 and #2 extracted from the fluorescence video (dashed line rectangle in (e)). The two colors in each trajectory correspond to "go" phases (in green for fND #1 and yellow for fND #2) and "stop" phases (in blue for fND #1 and red for fND #2), as identified by our trajectory analysis algorithm (see Methods); bottom: two-state ("stop" and "go") representation of the same trajectories over time: fND #1 moved faster than fND# 2 (scale bar: 1 s).

Velocity of fND-containing endosome was calculated as the length of the displacement vector over time from the first to the last point of a "go" phase, whereas the curvilinear distance and time separating these two points corresponded to run length and processivity, respectively. The diffusion coefficient *D* was calculated during the "go" phase, by fitting the mean-square displacement (MSD) to the quadratic equation $MSD(\Delta t) = v^2 \Delta t^2 + 4D \Delta t$, where *v* is the velocity. According to the Stokes-Einstein law, *D* is inversely proportional to the intracellular viscosity. During a "stop" phase, the pausing time was defined as the duration from the first to the last point, the pausing frequency was the number of "stop" phases per minute.

We checked that these readouts remained constant during the 45-minute recordings for each culture well (Supplementary Fig. 3), and that there were no significant differences between the readout values for two independent experiments (Supplementary Fig. 4). Mean values and other statistical properties of these parameters are provided in Supplementary Table 1. Apart from these quantitative readouts, it is worth underlying that the fND-tracking assay can also provide a superresolution mapping of microtubule bundles in freely developing neuronal branches. For example, the two trajectories of Fig. 1g are separated by \approx 100 nm, suggesting that the two fNDcontaining endosomes travel along two adjacent microtubules inside the same branch. Supplementary Fig. 5 and Supplementary Video 3 show another region in which four trajectories of endosomes can be seen in the same neuronal branch, no more than \approx 400 nm apart. Moreover, adjacent trajectories are separated by a distance of about 40-160 nm, which is consistent with reported values^{9,18}.

We first tackle the sensitivity of the technic using a pharmacological drug impacting the microtubule-based intraneuronal transport, at nanomolar concentrations. This transport is dependent on the microtubule (MT) cytoskeleton integrity, and MT can be depolymerized by extracellular application of nocodazole. Here, we used 2 nM and 5 nM nocodazole concentration during the course of the 1 h-experiment, whereas this drug is standardly applied at micromolar concentrations for several hours^{19,20}. As shown in Fig. 2, we observed (i) a decrease in velocity (p=3.8x10⁻⁴ and p=1.4x10⁻⁷ for 2 nM and 5 nM respectively), in run length (p=3.4x10⁻⁴, p=5.3x10⁻⁶) and in processivity (p=4.5x10⁻³, p=9.8x10⁻⁵), and (ii) an increase in pausing time (p=0.003, p=6.4x10⁻⁵). Conversely, we observed a decrease in pausing frequency only for 5 nM (p=0.016) and the diffusion coefficient were not significantly affected (Supplementary Fig. 6). The

increase in pausing time and decrease in run length and processivity are consistent with motors unable to overcome the microtubule interruption caused by depolymerization of the plus-end tips of microtubules. The constancy of the diffusion coefficient indicates that the viscosity is not affected inside neuronal branches. Altogether, intraneuronal transport parameters vary in a drug dosage-dependent manner and change as expected for MT depolymerization.



Figure 2 | Nanomolar sensitivity of the fND-tracking assay to nocodazole impairment of the microtubule cytoskeleton integrity. Cumulative probability densities of velocity, run length, processivity and pausing time, for control (black), concentrations of nocodazole of 2 nM (green) and 5 nM (red). Inset: bar plots presented as mean \pm s.e.m., which were calculated from the total number of trajectories. Figures written inside the bars represent the number of trajectories from *n*=4 embryos. Statistical significance: two stars (**) indicate a *p* value smaller than 0.01 and three stars (***) mean that *p*<0.001.

Next we evaluated if the fND-tracking assay is sensitive enough to reveal the impact of slight protein concentration changes (\approx 30%) found in brain of patients^{21,22} on the intraneuronal transport. We first assessed the sensitivity of the methodology to change in concentration of proteins directly bound to microtubule, such as TAU or MAP2. These molecules compete with molecular motors for MT binding sites, therefore acting as roadblocks ²³. Genetic risk factors regulating the attachment/detachment of MAPs, such as the protein kinase coded by *MARK1* gene, have long been associated to Alzheimer's disease^{24,25} and Autism Spectrum Disorders (ASD)²¹. We generated two *Mark1* transgenic mouse lines (Fig. 3a) leading to three distinct *Mark1* overexpression levels, namely line 38 (L38^{het}, heterozygotes), line 8 (L8^{het}, heterozygotes) and line 8 (L8^{hom}, homozygotes). We first measured the protein levels of Mark1 in WT, L38^{het} and L8^{het} animals (Fig. 3b, Supplementary Fig. 7a). L38^{het} and WT were not statistically different (8±4%, *p*=0.85), in contrast to L8^{het} (32±6%, *p*=0.011). Therefore, we decided to test if this slight ≈30% increase is sufficient to induce changes in intraneuronal transport

parameters, taking advantage of four different genotypes: WT, L38^{het}, L8^{het} and L8^{hom} (Fig. 3c). The Mark1 gene overexpression in L8^{hom} was measured by RT-QPCR to be twice that in L8^{het} ($42\pm5\%$ for L8^{het}, p=0.0013 and $87\pm15\%$ for L8^{hom}, p=0.0010). This measurement was done on the same primary cultures as the ones used for the fND-tracking assay, right after trajectories acquisitions.



Figure 3 | Sensitivity of the fND-tracking assay to subtle concentration changes of autismrelated Mark1 protein. a, Schematic representation of the *Mark1* transgene used to create transgenic mouse L38 and L8. b, Quantification of Mark1 gene overexpression levels. The protein levels of Mark1 in WT, L38^{het} and L8^{het} animals were measured by Western Blot. *Mark1* overexpression in WT, L8^{het} and L8^{hom} was evaluated by RT-QPCR (same neurons as the ones used in (c)). c, Effects of *Mark1* overexpression on velocity, run length and processivity intraneuronal transport parameters. d, Mechanistic model of the impact of Mark1 protein on intraneuronal transport, as described in Mandelkow *et. al.*²⁴ and consistent with our data. All data are presented as mean \pm s.e.m. (calculated from the total number of samples in (b) or of independent cultures in (c)). Figures written inside the bars represent the number of samples in (b) or the number of trajectories/independent cultures in (c). Statistical significance: one star (*) indicates *p* value smaller than 0.05, two stars (**) are for *p* <0.01.

Quantification of the intraneuronal transport dynamics revealed significant variations for L8^{het} and L8^{hom}, but not for L38^{het} in which no changes were observed in any of the readouts (Fig. 3c, Supplementary Fig. 7b). The latter is consistent with the absence of *Mark1* overexpression for this transgenic line, therefore acting as an internal control. In

L8^{het} and L8^{hom}, the monotonous increase in *Mark1* gene overexpression levels was accompanied by a corresponding increase in velocity (p = 0.03, p = 0.006 for L8^{het}, L8^{hom} respectively), in run length (p = 0.029, p = 0.022 for L8^{het}, L8^{hom} respectively) and in processivity (p=0.037 for L8^{hom} respectively). We also obtained a decrease in pausing frequency for L8^{het} (p=0.044). Altogether, intraneuronal transport parameters vary in a gene dosage-dependent manner and they change as expected for Mark1-mediated regulation of the microtubule track hindrance (Fig. 3d)²⁴. We therefore demonstrated the sensitivity of our assay to detect microtubule roadblock impairments linked to brain diseases, for protein concentration changes as small as $\approx 30\%$.



Figure 4 | Sensitivity of the fND-tracking assay to subtle overexpression of autism-related Slc25a12. **a**, Schematic representation of the *Slc25a12* transgene used to create transgenic mouse line L2. **b**, Quantification of *Slc25a12* overexpression in L2^{het} through its Slc25a12 protein level (using Western Blot). **c**, Effects of *Slc25a12* overexpression on velocity, run length and processivity intraneuronal transport parameters. All data are presented as mean \pm s.e.m. (calculated from the total number of samples in (**b**) or of independent cultures in (**c**)). Figures written inside the bars represent the number of samples in (**b**) or the number of trajectories/independent cultures in (**c**). Statistical significance: one star (*) indicates *p* value smaller than 0.05.

Finally, we evaluated if the fND-tracking assay is sensitive enough to detect the effect of subtle concentration change of one protein that do not belong to microtubule complexes but is indirectly involved in the intraneuronal transport via energy supplies. We focused on the psychiatric risk factor *SLC25A12* that is required for the mitochondrial integrity and found associated with different types of ASD^{26,22} and with Schizophrenia²⁷. We generated one *Slc25a12* transgenic mouse line (L2^{het}) mimicking the slight overexpression of this gene as found in brain tissues of ASD patients^{22,28} (Fig. 4a). We measured Slc25a12 protein levels in WT and L2^{het} and found a significant increase in L2^{het} ($32\pm7\%$, p=0.043) (Fig. 4b, Supplementary Fig. 8). The quantification of the intraneuronal transport dynamic revealed significant variations with a decrease in run length (p=0.022) and processivity (p=0.012) (Fig. 4c). This result demonstrates the sensitivity of the tracking assay to detect indirect intraneuronal transport perturbations, for protein concentration changes as small as $\approx 30\%$.

In this Letter, we have reported a hitherto unexplored approach to tackle the changes of intraneuronal transport parameters induced by brain disease-related genetic risk factors. Our methodology relies on fluorescent nanodiamond tracking inside freely developing branches of living neurons. The high brightness, perfect photostability and absence of cytotoxicity of fNDs make it possible to perform high throughput intracellular transport analysis. As a proof-of-principle, we established transgenic animals mimicking the subtle changes of protein concentrations (\approx 30%) found in brain of patients. The fND-tracking assay was sensitive enough to detect modifications of the intraneuronal transport parameters in the transgenic neurons. To the best of our knowledge, this is the first direct measurement of the functional impact of brain disease-related genetic risk factors using a nanoparticle-based methodology.

In a near future, with the swift development of the CRISPR/Cas9 genome editing technology²⁹, it is possible to model multiple brain disease-related genetic risk factors in transgenic animals. Our fND tracking methodology is adapted to understand how these multiple genetic variants interact with each other to impair the intraneuronal transport. Furthermore, powerful methodologies to dissect the molecular basis of complex brain diseases are emerging such as 3D-human neuron cultures derived from induced pluripotent stem cells obtained from patients^{30,31}. Our fND-tracking assay can be instrumental develop an unbiased diagnosis neuropsychiatric to of and neurodegenerative diseases, opening the door to applications in translational nanomedicine.

Methods

Hippocampal neuron cultures and fNDs internalization. Hippocampi from E18.5 mouse embryos were isolated, chemically and mechanically dissociated, before being plated in LabTekII chamber (see Supplementary Methods). fNDs internalization was conducted at DIV15/16. The maintaining medium was set aside and replaced by 0.5 ml of Neurobasal phenol-free medium (Invitrogen) to which we added 5 µl of \approx 30 nm-sized fND stock solution, reaching a final concentration of 5 µg/ml. After 10 min incubation, the medium was replaced by the old maintaining medium and neurons were placed 20 min in the incubator before starting the video acquisition.

Video processing and trajectory analysis

We recorded videos of total duration of 120 s, at a time resolution of 50 ms/frame with Nikon NIS software in the proprietary ND2 format (Fig. 1a). A filtering pretreatment was done with Fiji software (version 1.46j; NIH, USA), which consists in subtracting to each frame the median intensity of the whole stack in order to remove static fluorescent spots. For each dynamic fND-containing endosome, we reconstructed the full trajectory using the Spot Tracker plugin of ICY software (GPLv3 open source) developed by the Quantitative Image Analysis team at Institut Pasteur (Paris, France) ^{32,33}. Using the Track Manager plugin, we exported a text file containing the spot (X,Y) coordinates and the signal amplitude and background (based on a 2D-gaussian fitting of fluorescence spots), and we processed these data with a home-made program written for Igor Pro software (WaveMetrics Inc., USA). Detection of "stop" and "go" phases relies on two main steps, namely a coarse "stop" phase detection based on the confinement ratio function, and a refine "go" detection based on three different sequential filters as detailed in Supplementary Fig. 1 and S2. Transport readouts for each trajectory are then computed and the program finally makes a statistical report for the entire run set corresponding to one experiment, namely one LabTek well. The IgorPro program is available on request.

Localization precision

We calibrated the localization precision of our set-up by tracking the position of more than 100 fNDs immobilized on a glass coverslip and imaged over 100 frames. We then analyzed the video with the same tracking software parameters as for the intraneuronal trajectories. Movies were background-subtracted using Fiji plugin with a rolling ball of 20 pixels, in order to mimic the effect of a median subtraction. For each fND, the localization precision was measured as the standard deviation of the center positions of the fluorescent spot over time. We then plotted all localization precisions with respect to the signal-to-background ratio (SBR) associated to each fND (Fig. 1c). SBR was determined as the ratio of the maximum fluorescence intensity of the fND (amplitude minus residual background) over the residual background intensity. Therefore, trajectories containing more than 30% of points having a SBR smaller than 3 were not considered, so that according to the calibration, we achieved a localization precision better than 30 nm.

Transgenic mice constructs. We used a 3,200 bp mouse CamKII α and a regulatory sequence of 711 bp intron and polyA SV40 in a pGL3-basic vector. The pGL3-modified vector was realized in two steps: (i) the regulating sequence "intron+polyA SV40"

(711 bp) was amplified by PCR from pCDNA1.1 vector with the following primers: forward 5'-TATATCTAGACCCGGGTGGACAAACTACCTACAGAG (XbaI site underlined) and reverse 5'-TATA<u>GGATCC</u>GCGGCCGCAGAAATATGGCGTTGATACC (BamHI site underlined); (ii) the specific promoter "mouse minimal promoter CamKII α " (3,200 bp) was amplified by PCR from mouse brain cDNA with the following primers: forward 5'-TATAGCTAGCGAGATTCTTCCAGCTAGTTC-3' (NheI site underlined) and reverse 5'-TATAAAGCTTCCGTGCTCCTGAGTGCAAAC-3' (HindIII site underlined). The XbaI/BamHI and then the NheI/HindIII digestion products of the amplicon were inserted into the two multiple cloning site of the pGL3 basic vector. *E. coli* DH5a cells were co-transformed with these constructs. The coding regions of the mouse Mark1 gene (2,386 bp) and mouse *Slc25a12* gene (2,034 bp) were amplified by PCR (sequences of primers and probes are available on request. The two HindIII/Smal digestion products of the amplicons were inserted into the multiple cloning site of the pGL3 modified vector. E. *coli* DH5 α cells were co-transformed with this shuttle construct. Transgenes were injected in C57/Bl6 fertilized eggs for random integration into the moue genome (CNRS SEAT/TAAM UPS 44, Villejuif, France). See Supplementary Methods for embryo genotyping, RT-QPCR and Western Blots.

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Authors contributions

SH, FT and MS conceived and designed the experiments; SH, NM, YLM, AMLB, MPA, SM, XLL, JV, CP and RD performed the experiments; SH, NM, SM, MPA and XLL analyzed the data; PK, FJH, CCW, BP, YH, CS, AC, and HCC contributed materials/analysis tools; SH, FT and MS wrote the paper.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at <u>www.nature.com/reprints</u>. Correspondence and requests for materials should be addressed to F.T. or M.S.

Competing financial interests

The authors declare no competing financial interests.

Chromosome 21 large-scale yeast two hybrid screening uncovers chromatin and synaptic complexes involved in intellectual disabilities and related to Late Onset Alzheimer disease

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ABSTRACT

Intellectual disability (ID) found in Down syndrome (DS) that is characterized by an extra copy of ~477 genes on chromosome 21 is far to be well understood. In this study, we used two DS mouse models that either display an extra copy of Dyrk1A (mBAC-Dyrk1A) or of the mouse chromosome 16 syntenic region [Dp(16)1 Yey]. Exome sequencing of transcripts deregulated in hippocampus and mass spectroscopy proteomics uncover enrichment in GO: 0000786 nucleosome and GO: 0045202, synapse, indicating a chromatin and a synaptic deregulation in each of these two models, respectively. DYRK1A interacted directly with either CREBPP or EP300 in neuron nuclei. Furthermore, DYRK1A dosage deregulation indirectly induced changes in expression of genes encoding presynaptic proteins with a specific impairment of the non-NMDA long-term potentiation between hippocampal dentate gyrus and CA3 region. Using a large scale yeast two-hybrid screen of human brain library with human chr21 baits, we identified that chr21 encoded proteins (DYRK1A, DSCAM, TIAM1, KCNJ6, HUNK, GRIK1 and ITSN1) interacted with proteins involved in synaptic plasticity (DLG1-DLG4, SEPT7, GRIN2A/B, DLGAP1, SYNPO and AGAP3). Chr21 proteins display significant a specific pattern of positive selection as compared to random genes. They interact more frequently that random with proteins involved in ID (EP300, CREBPP, DYRK1A, HCN1, GRIN2A/B, KVNQ2 and GDI1) and Late-Onset Alzheimer Disease (LOAD) (APBA2, RIMBP2, BIN1 and CLU). Together, our data uncover previously unidentified interactions of chr21 proteins with key proteins involved in synaptic plasticity, but also with proteins directly involved in ID and LOAD, emphasizing links between these diseases.

INTRODUCTION

Down's syndrome (DS) is the most common form of intellectual disability (ID), affecting 1/700 births and results from one of the most complex genetic perturbations that is compatible with survival. DS is a human genetic disorder, induced by the presence of a third copy of up to ~477 genes from human chromosome 21 (Hsa21). In spite of a broad spectrum of clinical phenotypes, features consistent in all DS are the intellectual deficit that impairs learning and memory and an increased risk of developing a dementia very similar to Alzheimer's disease (AD) (Dierssen, 2012; Wiseman et al., 2015). However, the precise contribution of each Hsa21 protein overexpression to the cognitive impairment found in DS remains undetermined.

We focused on two mouse models of trisomy 21, a triplication of Dyrk1A based on a Dyrk1a BAC (189N3) (Guedj et al., 2012) and the Dp(16)1Yey model that displays a triplication of the 22Mb chromosome 16 syntenic region of Hsa21 corresponding two XXX genes syntenic to Hsa21 (Yu et al., 2010). DYRK1A BAC model displays modifications in synaptic plasticity both in hippocampus and in prefrontal cortex (Ahn et al., 2006; Thomazeau et al., 2014). Normalization of DYRK1A levels in Ts65Dn indicated a partial recovery of both synaptic plasticity and episodic memory (Altafaj et al., 2012). Together, these results indicate that synaptic plasticity changes and related episodic spatial memory defects depend on DYRK1A but also on other Hsa21 trisomy genes and/or non-Hsa 21 deregulated genes contribute to both hippocampal

The present study examined the respective contribution of DYRK1A and other Hsa21 gene products in pathways linked to intellectual disability (ID). Exome sequencing of transcripts deregulated in hippocampus and mass spectroscopy proteomics uncover enrichment in chromatin and synaptic repertoires deregulation in 189N3 and Dp(16)1 Yey, respectively. DYRK1A interacted directly with either CREBPP or EP300 that are chromatin remodelers in neuron nuclei. DYRK1A dosage deregulation indirectly induced changes in expression of genes encoding presynaptic proteins with a specific impairment of the non-NMDA long-term potentiation between hippocampal dentate gyrus and CA3 region.

Using a large scale yeast two-hybrid screen of human brain library with Hsa21 baits, we identified that Hsa21 encoded proteins interacted with proteins involved in synaptic function, linking DS with synaptopathies. Hsa21 proteins display a particular pattern of significant positive selection as compared to random genes. These Hsa21 proteins also interact more frequently that random with proteins identified as involved in ID and Late-Onset Alzheimer Disease (LOAD), indicating that multiple distinct pathways involving Hsa21 proteins contribute to cognitive endophenotypes and to the dementia that is found in DS as early as 40 years.

These results revealed previously unappreciated pathways involving chromosome 21 gene products and their impact on synaptic plasticity with possible consequences for ID and LOAD, emphasizing links between these diseases.

RESULTS

Whole-genome RNA sequencing and quantitative proteomics reveal two contrasted networks of deregulated genes of hippocampus from 189N3 DYRK1A and Dp(16)1Yey DS models

We used two DS mouse models; the 189N3 and the Dp(16)1Yey models. The 189N3 model is based on the insertion of a mouse BAC including the Dyrk1A gene, inducing a functional triplication of Dyrk1A (Guedj et al., 2012). The Dp(16)1Yey model displays a triplication of the 22Mb chromosome 16 syntenic region of Hsa21 that contains 115 Hsa21 gene orthologs including Dyrk1a (Yu et al., 2010). In contrast, the most studied Ts65Dn model is partially trisomic for the mouse chr16 region syntenic of Hsa21 but also includes a triplication of at least 60 non-DS-related genes of chr17 that complicate functional analysis (Duchon et al., 2011; Reinholdt et al., 2011). The Dp(16)1Yey model reflects precisely the gene dosage of Hsa21 orthologues but no genome-wide information was yet available on the deregulation of transcripts and their isoforms.

We first performed RNA sequencing on embryonic E17 hippocampi of these two DS models. We identified 84 deregulated genes in 189N3 mice (Supplementary Table S1) and 142 deregulated genes in Dp(16)1Yey (Supplementary Table S2) compared to their littermate controls. Gene ontology and gene network analyses of differentially expressed genes revealed a deregulation of chromatin proteins for 189N3 mice (Figure 1A&C). In contrast, Dapple analysis of Dp(16)1Yey evidenced a network with statistically significance for direct edge count (p<0.05) (Supplementary Fig. XX). This network includes proteins involved in synaptic plasticity (NRGN, SCG2, NGEF, CAMK2A and GRIN3A), abnormal neuronal projections (EPHA8, CLSTN3) and enhanced abnormal compulsive behavior (DLGAP3). This result prompted us to analyze protein changes in adult Dp(16)1Yey hippocampi using mass spectrometry. We evidenced changes in GO: 0045202 synapse component (Figure 1B&C). Interestingly, analysis of downregulated proteins gives as first significant category the same GO: 0045202~synapse (p=7,68E-006) with SYT1, CLSTN1, RIMBP2, LIN7C, ABI1, CDK5, PPP1R9B, SLC1A2, SLC1A3, GRM2, SYN1, GRIA1, SYN2, PRKAR1A, NTRK2, CYFIP1, VAMP2, SV2A, BIN1 proteins. The upregulated repertoire for GO: 0045202~synapse (p=0.001) includes SCAMP1, SNAP91, GABRA1, SYNGR1, PCLO, ITPR1, LIN7A, CTNNB1, SLC32A1, GRIA2, DLG4, CYFIP2, PEBP1, SV2B, CAMK2A. Altogether, these results indicate a contrasted deregulation of chromatin-related genes for Dyrk1A model and synapse proteins for Dp(16)1Yey model.

Hsa21 and positive selection

Some genes that display derived alleles with long haplotypes are candidates to a recent selective sweep (Sabeti et al., 2002). These alleles can be detected using the long haplotype test developed by Sabeti et al, 2002. We took advantage of recent updates of the 1000G dataset (Sudmant et al., 2015) to analyze three distinct populations, Africans (AFR), Asians (ASN) & Europeans (EUR). For Hsa21 GRIK1 locus that displays long haplotype for the derived allele in AFR, analysis centered on rs363517 showed a major derived haplotype when a variety of diverse ancestral haplotypes are evidenced (Figure 2B). Taking in account the three populations, we found 56 genes carrying at least one SNP with longer derived haplotype compared to ancestral, haplotypes including protein coding genes, long non coding RNAs and miRNAs (Figure 2C). From the Human Brain Transcriptome data (Kang et al., 2011), 36 genes (from 56) can be analyzed and showed high expression in striatum, hippocampus and amygdala regions at two precise developmental stages: late fetal time and adolescence time, respectively.

Establishment of a Hsa21 protein-protein interaction map by a high-throughput, protein domainbased yeast two-hybrid (Y2H) screening

We selected 51 human proteins based on their putative role in DS and its associated ID and screened a highly complex random-primed adult brain cDNA library comprising 10 million independent fragments in yeast. To ensure reproducible and exhaustive results, the library was screened to saturation thanks to an optimized mating procedure that allowed for testing on average 96 million interactions per screen, corresponding to a 9-fold coverage of the library. Moreover, multiple independent fragments of the same interactant could be isolated, enabling the delineation of a minimal interacting domain and the computation of a confidence score. This resource involves 5x10⁹ potential interactions from a brain prey library that is one order of magnitude more than the present reports of systematic map of 14,000 high-quality human binary protein-protein interactions. Although no bias is observed in the bait selection, analysis of the data suggests an enrichment of preys involved in specific molecular processes such as regulation of transcription, chromatin modification, ubiquitination and cell adhesion (**Supplementary Figure SXX**).

Interaction of Hsa21DYRK1A with chromatin remodelers CREBBP and EP300

DYRK1A is an important candidate to learning and memory impairment seen in DS patients (Smith et al., 1997) but regulatory pathways impaired by trisomy of DYRK1A remain elusive.

We focused on DYRK1A interactome based on our results (71 interactions) and recently reported data from Varjosalo et al., 2013. We first validated the interaction between RNASEN (alias DROSHA) and FAM53C in primary neuron nuclei (**Supplementary Figure S2 A-G**). RNASEN and DGCR8 which anchors RNASEN process miRNA transcripts into precursor miRNAs transcripts (Han et al., 2006; Lee et al., 2003). FAM53C is a nuclear protein of unknown function.

We next studied Y2H identified interaction between DYRK1A and EP300 or CREBBP. EP300 and CREBPP are the two members of the p300-CBP coactivator family. These two genes share encode histone acetyltransferases (HATs) that are involved in chromatin remodeling. Mutations in *CREBBP* and *EP300* genes have been identified to cause Rubinstein-Taybi Syndrome RTS (Bentivegna et al., 2006; Coupry et al., 2002). First we found DYRK1A in immunoprecipitates of HEK293 cells using antibodies against EP300 and CREBBP respectively (**Figure 3A-B; Supplementary Figure S3C**). We also validated direct nuclear interactions in primary cortical neurons using PLA (**Figure 3C; Supplementary Figure S4A**). *DYRK1A* gene dosage induced modifications the number of Dyk1a-Crebbp and Dyrk1a-Ep300 interactions whereas downstream interactions like Crebbp-Smarca2 and Ep300-Smarca2 were not modified (**Figure 3D; Supplementary Figures S4B**). These results are in full agreement with the involvement of DYRK1A in the REST/NRSF–SWI/SNF chromatin remodeling complex that we previously reported (Lepagnol-Bestel et al., 2009).

Non-NMDA dependent long term potentiation at the dentate gyrus-CA3 hippocampal synapse is abolished in Dyrk1A mouse models

As *CREBBP* and *EP300* encode HATs and impact chromatin remodeling, one can hypothesize that changes in number of interactions between DYRK1A and these HAT partners can consequently modify the transcription of genes involved in synaptic function (Alarcón et al., 2004; Guan et al., 2002; Kandel, 2001; Levine et al., 2005). As *Dyrk1A*, *Ep300* and *Crebbp* display high levels of expression in dentate gyrus, CA1 and CA3 hippocampus subregions (**Figure 3E**), we analyzed synaptic plasticity at different synapses of hippocampus and found abnormal synaptic potentiation in two

mouse models of trisomy 21, BAC 189N3 and YAC 152F7. In contrast to BAC 189N3 which overexpressed only mouse *Dyrk1a* (Guedj et al., 2012), YAC 152F7 incorporated a ~400kb human genomic fragment that includes *DYRK1A* but also *TTC3*, *PIGP*, *DSCR9* and *DSCR3* (Smith et al., 1997). Interestingly 152F7 mice were found to have a normal LTP in CA3-CA1 hippocampal synapse but an impaired spatial learning assessed by Morris water maze test (Smith et al., 1997). In both models, we found that the non NMDA dependent long term potentiation (LTP) was absent in the dentate gyrus-CA3 synapse (Figure 3F-G). This LTP is known to depend on molecular deregulations linked to the presynapse and is impaired in knockout mice where Rims1, Munc13-1 and Rab3a have been respectively invalided (Castillo, 2012). Using quantitative in situ hybridization and RT-Q-PCR from laser-assisted micro-dissection of hippocampal sub-regions, we were able to found that Rims1, Munc13-1 and Rab3a were decreased in 152F7 dentate gyrus (Figure 3H-I; Supplementary Figure S5). These results are in agreement with the deregulation of p300-CREBBP complexes by abnormal *DYRK1A* dosage that can modify the expression of genes encoding proteins involved in the molecular mechanism leading to presynaptic vesicle liberation such as Rims1, Munc13-1 and Rab3a (Südhof, 2013).

Interactome of Hsa21 proteins located in either dendritic spine heads or necks

As we found an enrichment in synaptic proteins in Dp(16)1Yey transgenic mouse model (**Figure 1B&C**), we analyzed the subcellular localization by proximity ligation assay (PLA) of Y2H identified interactions of the the cytoscape network (**Figure 4A**).

First, we identified of Dscam with Dlg2 and Dlg4 in dendritic spines (**Figure 4B**). One-by-one Y2H approach identified interactions between DSCAM and the four members of DLG family DLG1, DLG2, DLG3, DLG4 (Supplementary Fig. XX). DSCAM is known to regulate dendrite arborization and spine formation during cortical circuit development (Maynard and Stein, 2012). DLG1 (alias SAP97), DLG2 (alias PSD93/chapsyn-110), DLG3 (alias SAP102) and DLG4 (alias PSD95/SAP90) are known to bind to various proteins and signaling molecules at the postsynaptic density (PSD) (Sheng and Kim, 2011). Furthermore, mutations of DLG2 and DLG4 induce distinct cognitive phenotypes in both human and mice (Nithianantharajah et al., 2013). DSCAM also interacted with DYRK1A via a common domain shared by DSCAM, its human paralogue DSCAML and in its Drosophila orthologue Dscam4 (**Supplementary Figure S2D-F & S3B**). We could hypothesize that conservation of this protein-protein interaction through species is consistent with roles of DSCAM similar through species.

Hsa21 ITSN1 interacted with SNAP25 and DLGAP1 in dendritic spines. SNAP25, is a member of the SNARE protein family essential for the exocytosis of synaptic vesicles (Südhof, 2013) but is also involved in trafficking of postsynaptic NMDA receptors (Jurado et al., 2013) and spine morphogenesis (Tomasoni et al., 2013). DLGAP1 (alias GKAP) is a core protein of the scaffolding complex of the synapse (Naisbitt et al., 1999). These results are in full agreement with Itsn1 mutant mice phenotype that is characterized by severe deficits in spatial learning and contextual fear memory (Sengar et al., 2013).

Hsa21 KCNJ6, a voltage-insensitive potassium channel member of the GIRK family, interacted with three Dlg members, i.e. Dlg1, Dlg2 and Dlg4 (**Figure 4E**). We also found an increase of Kcnj6-Dlg2 interactions in Dp(16)1Yey transgenic mouse model compared to control. In contrast, the number of Grin2ab-Dlg2 interactions did not change (**Figure 4H**; **Supplementary Figure S4C**). KCNJ6 is expressed in dendrites and dendritic spines at the PSD of excitatory synapses (Luján et al., 2009) and its trisomy induces synaprtic and behavioral changes (Cooper et al., 2012). Intriguingly, mice lacking *Dlg2* and people with *DLG2* mutations had abnormal cognitive abilities (Nithianantharajah et al.,

2013). One can propose that these phenotypic changes found in mice trisomic for Kcnj6 are generated via deregulations involving DLG2.

HUNK (alias MAK-V) was found to interact with AGAP3 and SYNPO, using PLA (**Figure 4F**). Synpo localized in necks of dendritic spines and linked to the spine apparatus assuming an essential role in regulating synaptic plasticity (Segal et al., 2010). We also identified a new interaction with Agap3, a GTPase-activating protein recently identified as an essential signaling component of the NMDA receptor complex that links NMDA receptor activation to AMPA receptor trafficking (Oku and Huganir, 2013). These results suggest that HUNK is involved in pathways localized both near PSD95 complexes and spine apparatus.

We next focused on protein-protein interactions implying Hsa21 *GRIK1* gene product, one of the kainate ionotropic glutamate receptor (GluR) subunits that function as a ligand-gated ion channel. Kainate receptors (KARs) are found ubiquitously in the CNS and are present pre- and post-synaptically (Lerma & Marques, 2012). We first validated GRIK-KCNQ2 interaction in dendritic spine (**Figure 4G**). As KCNQ2 channels are known to functionally interact with HCN1 channels in prefrontal cortex dendritic spines (Arnsten et al., 2012), we tested if HCN1, KCNQ2 and GRIK1 physically interact in dendritic spines using PLA and found a direct interaction between GRIK1 and HCN in dendritic spines (**Figure 4G**). We also validated interactions of GRIK1 with SEPT and KALRN (**Figure 4H**). SEPT7, a member of the septin family of GTPases is localized to dendritic branching points and spine necks (Tada et al., 2007; Xie et al., 2007), regulating membrane protein access to spines (Ewers et al., 2014). KALRN is a Rho-GEF exclusively localized to the postsynaptic side of excitatory synapses (Ma et al., 2003, 2008) and binds to NMDA receptor subunit Nr2b (Kiraly et al., 2011). These results indicate that GRIK1 is part of two synaptic complexes, one located near PSD95 (DLG4) at the tip of the dendritic spine and the other at the neck of spines.

Altogether, these results indicate that Hsa21 proteins are involved in distinct dendritic spine pathways located either in dendritic spine heads or in dendritic spine necks that are known regulate compartmentalization of synapses (Tønnesen et al., 2014).

Linking Hsa21 proteins to intellectual disabilities and to LOAD

We finally asked if interactions between chromosome 21 proteins and cognition diseases-related gene products from the protein-protein interactions map could be validated in sub-neuronal compartments, in particular in dendritic spines. The Hsa21 USP25-GDI1 interaction was identified by Y2H (Figure 5A and Supplementary Table XX). *GDI1* was one of the first X-linked-gene to be identified as involved in ID (D'Amato et al., 1998) and also in autism spectrum disorders (ASD) (O'Roak et al., 2012; Pinto et al., 2014). We also validated Hsa21 DYRK1A-STX1A and Hsa21 HUNK-LIMK1 interaction in dendritic sines (Figure 5B). Both STX1A and LIMK1 are encoded by genes located on Williams-Beuren Syndrome (WBS) ~1.5Mb deletion region whose mouse model reproduces cognitive traits including hypersociability (Li et al., 2009). More than 700 genes involved in intellectual disabilities have been recently identified (Vissers et al., 2015). Comparison of this repertoire as a function of total human genes (20313 human Ensembl coding genes) and the number of ID proteins directly interacting with Hsa21 reveals a statistically significant increase in Hsa21 interactors tested in neurons (p=3.9x10⁻³).

Links between DS and Alzheimer-related dementia remains largely unknown, in spite of the early onset of LOAD-like dementia evidenced as early as 40 years in DS (Wiseman et al., 2015). Triplication of APP have been proposed to be responsible for LOAD-like dementia but increased expression of wild-type APP, even at levels in excess of those present in Down syndrome (DS), is insufficient to

cause extensive Alzheimer disease (AD) neuropathology (Balducci and Forloni, 2011). Interestingly, RIMBP2 that was associated to LOAD displaying psychotic symptoms (Hollingworth et al., 2012) is deregulated in adult mouse hippocampus (Figure 1B). RimBP2 is involved in molecular machine for neurotransmitter release (Südhof, 2013). Different Hsa21 products interact with proteins involved in LOAD (Figure 5C). Hsa21 HUNK interacts with APBA2 that is a modulator of LOAD associated amyloid beta A4 precursor protein (APP) endocytosis and metabolism (Rhinn et al., 2013). Hsa21 TIAM1 interacts with BIN1 that is a risk factor from LOAD-GWAS studies (Lambert et al., 2013). BIN1 was also identified as linked to cognition in LOAD patients (Vivot et al., 2015). Furthermore, BIN1 genotype affects working memory, hippocampal volume, and functional connectivity in young healthy individuals (Zhang et al., 2015). Hsa21 TIAM1 has been proposed to be responsible in mice of synaptic abnormalities (Sala and Segal, 2014). Here we validated Tiam1-Bin1 interactions in dendritic spines, with a similar localization for Tiam1-Dlg1 interaction (Figure 5C; Supplementary Figure S6B). We also evidenced hsa21 DSCR9-CLU interaction (Figure 5C). DSCR9 and DSCR10 are genes exclusive to primate genomes such as chimpanzee, gorilla, orangutan, crab-eating monkey and African green monkey and are not present in other non-primate mammals (Takamatsu et al., 2002) (Supplementary Figure S7).

Together, these results indicate that chromosome 21 proteins interact with proteins previously identified as key molecules in cognition diseases as diverse as X-linked ID, WBS, ASD and LOAD dementia.

Discussion

In spite of various mouse models of DS available, cognitive impairment phenotypes found in DS have not been related to specific alterations of molecular pathways. Furthermore, to the best of our knowledge, no specific synaptic alterations have been described in models overexpressing a given chr21 gene as compared to models with overexpression of a syntenic region.

In the present study, we analyzed molecular changes in hippocampus of Dyrk1A BAC (189N3) and of the transgenic mouse model that integrated the entire Hsa21 syntenic region on Mmu16, Dp(16)1Yey. We found molecular changes linked to chromatin remodeling in the 189N3 mice. Both the 189N3 as 152F7 mice that have a DYRK1A centered YAC fragment (Smith et al., 1997) display a specific impairment of non-NMDA LTP in the Dentate Gyrus-CA3 synapse. This specific cognitionrelated phenotype is linked to the decrease of Rims1, Munc13-1 and Rab3a genes that are known to be involved in molecular machine for neurotransmitter release (Südhof, 2013). The related cognitive impairment appears to be rather characteristic with impaired spatial reversal learning (Smith et al., 1997, D'Adamo et al., 2004). In contrast, mouse model of DS (Tc1) that carries an almost complete Hsa21 including DYR1A do not have impairment in non-NMDA LTP in the Dentate Gyrus-CA3 synapse, in spite of changes in presynapse properties (Witton et al., 2015). Together, these results demonstrate that impairment of non-NMDA LTP in the Dentate Gyrus-CA3 synapse can be obtained only is DYRK1A alone is in three copies and that overexpression of other hChr21 genes impact these molecular mechanisms. Furthermore, these results indicate that impairment of Dentate Gyrus-CA3 synapse non-NMDA LTP depends on DYRK1A-dependent chromatin remodeling involving DYRK1A interaction with EP300 and CREBBP. This phenotype is a novel specific synaptic readout linked to DYRK1A overexpression. Consequences therapeutics not directly based on DYRK1A kiase properties but on downstream targets such as presynaptic components of the molecular machine for neurotransmitter release

In contrast, expression of an extra copy of the entire Hsa21 syntenic region on Mmu16 spans 22.9 Mb and contains 115 Hsa21 gene orthologs including DYRK1A in Gene Ontology neuron projection and synapse classes. Novel interactions with protein involved in postsynaptic complexes have been identified. Hsa21 GRIK1 was found to interact with cyclic nucleotide modulated ionic channels such as KCNQ2 and HCN1 ion channels that are known to be involved in epilepsy, working and spatial memories, respectively (Schroeder et al., 1998; Wang et al., 2007; Giocomo et al., 2011). Hsa21 GRIK1 also interacts with SEPT7 and KALRN. SEPT7 is located that is a specific protein of the neck of dendritic spines (Tda et al., 2007), suggesting that Hsa21 GRIK1 can be part of a complex located in the neck of dendritic spines. KALRN is a GDP/GTP exchange factor for Rac1, ere it regulates dendritic morphogenesis through Rac1 signaling to the actin cytoskeleton (Xie et al., 2007; Hayashi-Takagi et al., 2010). Signalosome complexes involving DISC1-KALIRIN-PSD95-RAC1 have been recently identified as able to modulate the RAC1 activity depending on the dosage of DISC1 (Hayashi-Takagi et al., 2010). Similarly, it is possible that protein-protein interactions identified here are part of different dendritic spine signalosome that are deregulated by three doses of Hsa21 proteins inducing modifications in the efficacy of these synaptic signalosomes.

In conclusion, our results provide the first report, to our knowledge, of differential impacts of chromosome 21 DYRK1A on chromatin remodeling and of the 115 Hsa21 gene orthologs including DYRK1A on synapse function. The contrasted pathways evidenced here are novel therapeutics targets for treating cognitive impairments found in DS. Furthermore, our results exemplify the link of DS with other forms of intellectual Disability and with degenerative disease with complex genetics such as LOAD.



Figure 1. Whole-genome RNA and protein sequencing in 189N3 and Dp(16)1Yey transgenic mouse models

A. Schematic representation of protein-protein interactions using Cytoscape software between the products of differentially expressed genes from the RNA sequencing performed on 189N3 embryonic E17 hippocampus implied in "chromatin assembly" GO DAVID class (Red circles).

B. Schematic representation of protein-protein interactions using Cytoscape software between the differentially expressed proteins from the Mass Spectometry sequencing performed on Dp(16)1Yey adult hippocampus implied in "synaptic transmission" GO DAVID class (Red circles).

C. GO DAVID class probabilities represented with –log(p-value). Red bars represented the first three GO DAVID classes of the differentially expressed genes from the RNA sequencing performed on 189N3 embryonic E17 hippocampus. Blue bars represented the first three GO DAVID classes of the differentially expressed genes from the RNA sequencing performed on Dp(16)1Yey embryonic E17 hippocampus. Grey bars represented the first three GO DAVID classes of the differentially expressed genes from the RNA sequencing performed on Dp(16)1Yey embryonic E17 hippocampus. Grey bars represented the first three GO DAVID classes of the differentially expressed genes from the Mass Spectometry sequencing performed on Dp(16)1Yey adult hippocampus.



Figure2. Analysis of selection pressure of chromosome 21

A. Histogram of number of genes in function of Omega value (Ka/Ks) for chr20 and chr21.

B. Bifurcation diagram in African population of SNP rs363517 (GRIK1 gene locus) exhibit long haplotype of derived allele compare to ancestral allele.

C. Venn Diagram by population of number of genes carrying at least one SNP with long derived haplotype compare to ancestral allele.



Figure3. Deregulation of synaptic nuclear protein-protein interactions in 189N3 transgenic mouse model and abnormal non-associative long-term potentiation (LTP) in transgenic 189N3 and 152F7 mice

A. Schematic representation of nuclear protein-protein interactions perfomed by yeast-two-hybrid using Cytoscape software

B. HEK293 cells were immunoprecipitated (IP) using anti-EP300 and anti-CREBBP antibodies and using anti-IgG antiboby as a negative control. The input and precipitated fractions were analyzed by western blot using anti-Ep300, anti-Dyrk1a and anti-Crebbp antibodies. The arrows indicate proteic bands at the expected size. Note that no cross-reaction was found with the IgGs.

C-D. *In situ* proximity ligation assays (PLA) on primary cortical neurons fixed at DIC7 (red fluorescence) using anti-Dyrk1a and anti-Ep300 or anti-Crebbp, anti-Smarca2 and anti-Ep300 or anti-Crebbp antibodies. Nuclear bodies were labelled using Topro3 staining (blue fluorescence). Mean interaction point numbers were calculated in nuclear body of 45 to 89 cortical neurons at DIC7 (from 3 to 5 different embryos per genotype). PLA using anti-Ep300 and anti-Fibrillarin antibodies were performed as a negative control and no difference was shown between transgenic 189N3 and WT cortical neurons. Scale bars=10 μ m. * p < 0.005; *** p < 0.0005.

E. False-color image of ISH from Allen Brain Atlas showing Dyrk1a, Ep300 and Crebbp transcript expression in adult mouse hippocampus. Scale bar = 100μm.

F-G. Summary (mean+/-s.e.m.) of mossy fibre LTP in transgenic 189N3 compared to WT mice (F1) and in transgenic 152F7 compared to WT mice (G). Distributions for the magnitude of LTP were observed in 189N3 and WT mice (F2). Recording were performed on 4 to 6 mice in each genotypes.

H. False-color image of antisense Rims1 RNA Q-ISH of hippocampus from juvenile P21 WT and 152F7 mice. Q-ISH quantification indicates a significant down-regulation of Rims1 in the three subregions of the 152F7 mouse hippocampus.

I. Laser-assisted microdissection of the three subregions of P21 mouse hippocampus (see Supplementary figure). Rims1, Syn2, Rab3a and Munc13-1 are down regulated in the DG and CA3 hippocampal subregions of juvenile transgenic 152F7 mice compared to their WT siblings as shown by Q-RT-PCR analysis. Scale bar = 1mm. *p<0.01**p<0.001. ***p<0.0001



Figure4. Synaptic protein-protein interactions and modification in Dp(16)1Yey transgenic mice. A. Schematic representation of synaptic protein-protein interactions performed by yeast-two-hybrid using Cytoscape software. Red circles correspond to genes localized on human chromosome 21. B-H. *In situ* proximity ligation assays (PLA) on primary cortical neurons fixed at DIC21 (red fluorescence) using anti-Dscam and anti-Dlg1 or anti-Dlg2 or anti-Dlg4 antibodies (B), anti-Itsn1 and anti-Dlgap1 antibodies (C), anti-Kcnj6 and anti-Dlg2 or anti-Dlg4 antibodies (D), anti-Hunk and anti-Agap3 antibodies (F), anti-Grik1 and anti-Hcn1 or anti-Kcnq2 antibodies (G), anti-Grik1 and anti-Sept7 or anti-Kalrn antibodies (H). PLA using anti-Dlg2 and anti-Grin2ab (B), anti-Itsn1 and anti-Snap25 (C), anti-Kcnj6 and anti-Dlg1 (D), anti-Hunk and anti-Synpo (F), anti-Grik1 and anti-Dlg4 (H), were performed as positive controls. PLA using either anti-Smarca4 or anti-Baf155 antibodies are

performed as negative control. Dendritic network and dendritic spines were labelled using phalloidin staining (green fluorescence). Mean interaction point numbers were calculated in dendrites of 25 to 30 cortical neurons at DIC21. Please see Supplementary Figure for negative controls.

E. *In situ* PLA on transgenic Dp(16)1Yey and WT primary cortical neurons fixed at DIC21 (red fluorescence) using anti-Dlg2 and anti-Kcnj6 or anti-Grin2ab antibodies. Dendritic network and dendritic spines were labelled using phalloidin staining (green fluorescence). Mean interaction point numbers were calculated in dendrites of at least 26 cortical neurons at DIC21 (from 3 different embryos per genotype). * p < 0.05

Scale bars=10µm.

A X-linked Intellectual disability



Figure5. Synaptic protein-protein interactions involving neurodevelopmental disease-related genes.

A. Synaptic protein-protein interactions involved in X-linked Intellectual Disability (ID). *In situ* proximity ligation assays (PLA) using anti-GFP and anti-HA or anti-MYC antibodies on HEK293 co-transfected cells with GFP-USP25 and HA-GDI, GFP-USP25 and MYC-SYK as positive control, GFP-USP25 alone as negative control (red fluorescence). Green fluorescent protein was visualized on green channel. Mean interaction point numbers were calculated in GFP-expressed compartments of at least 17 transfected HEK293 cells.

B. Synaptic protein-protein interactions involved in Williams Beuren Syndrom (WBS). Scheme of the genetic map for the chromosomic 1.5Mb region commonly deleted in WBS (upper panel). *In situ* PLA on primary cortical neurons fixed at DIC21 (red fluorescence) using anti-Dyrk1A and anti-Stx1A antibodies or anti-Hunk and anti-Limk1 antibodies. PLA using either anti-Dyrk1A and anti-Tomm20 or anti-Hunk and anti-Baf155 antibodies were performed as a negative control. Dendritic network and dendritic spines were labelled using phalloidin staining (green fluorescence). Mean interaction point numbers were calculated in dendrites of at least 29 cortical neurons at DIC21.

C. Synaptic protein-protein interactions involved in Late Onset Alzheimer Disease (LOAD). *In situ* PLA on primary cortical neurons fixed at DIC21 (red fluorescence) using anti-Hunk and anti-Apba2 or anti-Bin1 and anti-Tiam1 or anti-Dlg1 or anti-Dlg4 antibodies. PLA using anti-Tomm20 and anti-Bin1 or anti-Tiam1, anti-Hunk and anti-Baf155 were performed as negative controls. Dendritic network and dendritic spines were labelled using phalloidin staining (green fluorescence). Mean interaction point numbers were calculated in dendrites of 24 to 30 cortical neurons at DIC21. *In situ* PLA on primary cortical neurons transfected at DIC5 and fixed 48hours later at DIC7 (red fluorescence) using anti-GFP and anti-Fibrillarin antibodies were performed as a negative control. Green fluorescent protein was visualized on green channel and nuclear bodies were labelled using Topro3 (blue fluorescence). Mean interaction point numbers were calculated in GFP-expressed compartments of 30 transfected cortical neurons. Please see Supplementary Figure for negative controls.

Scale bars=10µm.

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AUTS2, a gene implicated in psychiatric and neurodevelopmental disorders is a modulator of AKT2 in dendritic spines

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AUTS2 was found disrupted in diverse psychiatric. neurological and neurodevelopmental disorders suggesting that its dosage contributes to molecular basis underlying social behavior and cognitive ability. AUTS2 locus displays a significant increase in the number of neuron-related transcription factor sites as compared to Neandertal AUTS2 locus. In spite of its potential dosage-sensitive contribution to neural pathways underlying social behavior and cognitive ability, the function of AUTS2 remains unknown. In primary neuronal cultures we found that AUTS2 protein localizes to dendrites and dendritic spines. We modeled AUTS2 haploinsufficiency via shRNA-mediated knock-down in mouse neurons, and observed robust alterations in dendritic spine morphology (length and width) and synaptic currents. We find that AUTS2 interacts locally at dendrites with TTC3, the E3 ubiquitin ligase for AKT2, a key kinase of dendritic spines. AUTS2 silencing induces robust alterations in synaptic currents that are rescued by presynaptic injection of AKT2 indicating that AUTS2 acts as a modulator of AKT2 in postsynaptic function.

Since the identification of AUTS2 translocation in a pair of autistic twins (1), a variety of rare genomic structural variations involving AUTS2 have been reported in ASDs (2-6), in mental retardation (7), in epilepsy (8) and in a variety of psychiatric diseases (9-10) (**Supplementary Table S1**). Furthermore, AUTS2 common variant was recently found associated to alcohol consumption using a GWAS approach (11). Consistent with its role in psychiatric and cognitive disorders, it has been shown that the first half of AUTS2 gene has undergone significant evolutionary divergence since the split between Neanderthals and present-day humans (12).

AUTS2 is expressed in the developing human brain (Figure 1A) and consistent with evidence for positive selection in early modern humans, the *AUTS2* locus displays a statistically significant increase in neuron-specific transcription factor sites as compared to Neanderthal *AUTS2* locus (Supplementary Figures S1; Supplementary Table S2). To analyze Auts2 protein function, we generated novel polyclonal antibodies raised against a peptide corresponding to N-terminal amino acid of the protein (see Material & Methods). In addition to nuclear (*13-15*) and dendritic staining (Supplementary Figure S2), mouse primary cortical and hippocampal cultures at day in culture (DIC) 21 showed a robust signal in dendritic spines (Figure 2A; Supplementary Figure S3). Auts2 was found enriched in adult mouse brain, adult mouse brain nuclear enrichment fraction and adult mouse cortical synaptosomal enrichment fraction (Figure 1B). Auts2 was localized in discrete puncta adjacent to anti-synapsin puncta and fully overlapping with PSD95 and SYNPO, indicating that AUTS2 may be a novel postsynaptic protein (Figure 1C). Super-resolution analysis of

Auts2 using d-STORM indicated that Auts2 is located in dendritic spines, with a statistically significant increase of clusters in the intermediate compartment of dendritic spine ("neck & head") (Figure 1D). Auts2 was also found enriched in this compartment for SYNPO+ dendritic spines (Figure 1E). Search for protein interactors was performed using yeast twohybrid (Y2H) screens and identified ~18 AUTS2 potential interacting partners including tetratricopeptide repeat domain 3 protein (TTC3) (Supplementary Figure S4). The function of TTC3 as ubiquitin E3 ligase specific for AKT (16) prompted us to further study this interaction. We confirmed the interaction between TTC3 and AKT2 using the one-by-one assay. AUTS2 interacts with TTC3 in a distinct domain located to the N-terminal part of the protein (1-481 amino acid domain that includes the first tetratricopeptide repeat (TPR) motif and including S378, a key AKT-dependent phosphorylation site (16) (Supplementary Figure S4). Furthermore, we confirmed the AKT2-TTC3 interaction at the same domain as described in (16) and identified a novel interaction between AKT2 and CITRON (rhointeracting, serine/threonine kinase, CIT). The AUTS2, AKT2 and CITRON interactions are of particular interest as both AKT and CITRON kinases regulate dendritic spine morphogenesis (17-22). We confirmed that these proteins directly interacted in dendrites of cortical neurons, using the in situ proximity ligation assay (PLA), which enables detection of endogenous protein-protein interaction events (23) (Supplementary Figure S5). Notably, AUTS2, TTC3 and CITRON are co-expressed in human embryonic brain subregions known to be involved in neurodevelopmental diseases (Supplementary Figure S6).

To investigate whether AUTS2 is involved in spine morphogenesis we used shRNA vectors to mediate gene knockdown (Supplementary Figure S7). We quantified the dendritic spine number as well as their length and width in mature cortical neurons transfected with Auts2 sh1 (Figure 2A). We focused on three classes of dendritic spines; filopodia, stubby and mushroom spines. We did not find any changes in the total spine numbers, and in numbers of different spine classes (Supplementary Figure S8) but observed a significant decrease of the mushroom (large diameter) spine length and width (Figure 2B). Morphological changes in the population of mushroom spines induced by Auts2 deficiency is an indication of abnormal spine maturation and synaptic function. We confirmed synaptic impairment by analysis of the electrophysiological properties of the same cells, demonstrating that spontaneous excitatory postsynaptic current (sEPSC) amplitude responses were impaired in Auts2 silenced postsynaptic neurons as compared to postsynaptic neurons transfected by scrambled shRNA (Figure 2C-D). In contrast, frequencies of sEPSC were not modified. Furthermore guantal analyses of sEPSC responses indicate a decrease in the elementary event, suggesting that Auts2 silencing in postsynaptic neurons induce a decrease in the elementary postsynaptic response that can be attributed to changes in properties of postsynaptic receptors. Auts2 silencing also impacts

the complexity of neuronal processes in both axons and dendrites (**Supplementary Figures. S8 & S9**). Taken together these data strongly suggests that *Auts2* deficiency such as the one observed in cases of disease-associated structural variants, may impact ultimately synaptic connectivity leading to abnormal function of excitatory synapses.

It was previously reported that TTC3 facilitated Akt degradation both in the nucleus and (albeit less efficiently) in the cytoplasm of 293T cells (16), suggesting that the size of the AKT pool in a subcellular compartment can be modulated via TTC3-mediated degradation. AUTS2 may limit the activity of TTC3 by sequestering it. Therefore AUTS2 downregulation may induce an increase in TTC3 E3 ubiquitin ligase activity, leading in turn to a decrease of the AKT2 pool in the dendritic spine subcompartment. Consistent with this expectation we detected, using PLA as a direct readout, a decrease in the number of TTC3-AKT2 complexes upon Auts2 depletion [mean interaction points, a measure of proximity between Ttc3 and Akt2 was 38+7 (n=18) for control (sh-NS) and 22+4 (n=25) for Auts2 sh1 (p=0.0357), Figure **3C**]. We hypothesized that the observed decrease in synaptic current amplitude induced by Auts2 depletion was due to a local depletion of AKT2 molecules. Accordingly, we tested if injection of AKT2 into Auts2-deficient cortical neurons could rescue the altered synaptic phenotype. As before, we observed that Auts2 knockdown was associated with a decrease in the mean EPSC amplitude (non transfected: -47 ± 2 pA; GFP alone: -43 ±3 pA, Auts2-/GFP: 28 \pm 2 pA, p<0.05 as compared to control groups, n=7, 3 and 5 cells respectively). To rescue this phenotype, we introduced purified native or inactivated AKT2 protein (7.5 nM) through the patch pipette, while measuring the amplitude of excitatory spontaneous currents (Figure 3 D-E). First we established that under control conditions, the long lasting recordings did not introduce changes in sEPSC mean amplitude. Indeed, in both non transfected neurons recorded with pipettes filled by inactivated AKT2, and in GFP-expressing cells infused with native AKT2, mean sEPSC amplitude recorded >40 minutes after achieving whole cell configuration did not differ from initial sEPSC amplitude measured 2 minutes after the seal opening (non transfected: 0-10 min: -43 ± 3 pA, 40-50, min: -42 ± 4 pA; GFP alone: 0-10 min: -47 ± 2 pA, 40-50, min: -39 ± 6 pA, p>0.05). In contrast, if different at the seal opening, the mean sEPSC amplitude in Auts2 cells remained unchanged 40-50 minutes after seal opening, because of progressive increase of sEPSC amplitude in these cells (Auts2-/GFP: 0-10 min: 28 ± 2 pA, 40-50, min: -39 ± 6 pA, p>0.05 as compared to control groups at 40-50 min). Importantly, upon heat denaturation, AKT2 had no effect when introduced into Auts2-defcient cells (0-10 min: $-18 \pm 1 \text{ pA}$; 40-50 min: $17 \pm 2 \text{ pA}$, p>0.05, n=5 cells, data not shown). Overall, introduction of nanomolar concentrations of AKT2 into Auts2-deficient neurons, reversed the size of miniature postsynaptic currents to wild-type levels in a time period compatible with the trafficking of AKT2 from the soma to dendritic spines. Taken

together, these results suggest that AUTS2 acts as a modulator of the ubiquitin E3 ligase TTC3 that regulates the levels of AKT2 kinase in dendritic spines.

AKT signalling has been implicated both in spine formation and excitatory glutamatergic transmission (*24-26*). Therefore we propose that the effects of *Auts2* on dendritic spines and excitatory synapses may stem, at least in part, from modulating locally the number of AKT2 molecules and the duration of AKT2 action, impacting the excitatory transmission. The involvement of the AKT signaling pathway has already been demonstrated in schizophrenia (*27-28*). Our results raise the possibility that reduced AUTS2 gene dosage impairs the PI3K/AKT in a number of other neuropsychiatric and neurodevelopmental diseases, contributing to the abnormal function of neural pathways underlying social behavior and cognitive ability that characterize such disorders.

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Supporting Online Material

Materials and Methods Figs S1 to S9 Tables S1 to S2

Figures



Figure 1: AUTS2 expression during human brain development and dendritic spine localization of mouse Auts2 protein

A. Coronal sections of 8-week-old, anterior and posterior coronal sections of 22-week-old human brain embryos hybridized with AUTS2 antisense radioactive riboprobe. Quantification indicating expression of AUTS2 both in cortex and hippocampus at 22-week-old human embryonic development with a significantly higher expression level in cortical regions (AC: anterior cortex; PC: posterior cortex) than in hippocampus (HPC). Scale bar=1mm. *=p<0.05

di: diencephalon; ic: internal capsule; fr c: frontal cortex; ge: germinal zone; hip: hippocampus; in: insular cortex; mes: mesencaphalon; nc: nucleus caudate; nl: nucleus lenticular; rho: rhombencephalon; rl: rhombic lip; tc: temporal cortex; tel: telencephalon.

B. Fluorescent immunoblot expression of Auts2 protein (green band) in adult mouse brain, adult mouse brain nuclear enrichment fraction and adult mouse cortical synaptosomal enrichment fraction. Enrichment purity was controlled with PSD95 protein expression (red band).

C. Confocal imaging of immunocytochemistry on DIC21 primary hippocampal neurons using AUTS2 antibody and the postsynaptic markers PSD95 and SYNPO. Enlargements of dendritic spines images are shown. Pearson's coefficient Rr = $27.1\% \pm 1.6\%$, Rr = $24.8\% \pm 1.1\%$, respectively; n=21 and n=22.

(Scale bars=10 μ m). As positive control, Pearson's coefficient for Psd95-Homer and Psd95-Synpo are Rr = 36.7% ± 0.9% and Rr = 38.7% ± 2.1% respectively (n=21 and n=22). Scale bar=10 μ m.

D. Super-resolution dSTROM imaging of immunocytochemistry on DIC21 primary cortical neurons using AUTS antibody (white dots) and conventional imaging of phalloïdine (green). Histogram representing Auts2 cluster per dendritic spines, with in average 4,1 \pm 0.3 clusters/spine. Cumulative probability density of the number of Auts2 cluster in neck (salmon), neck&head (purple) and head (green). Inset: bar plots show the average of Auts2 cluster per spine sub-region, with 3.6 \pm 0.2 clusters in the head, 3.5 \pm 0.5 clusters in the neck and 7.1 \pm 0.6 clusters in the entire spine (neck&head). Figures inside bar plots represent the number of samples. Localization of Auts2 clusters inside the spine head (green), the neck (salmon) or both of them (grey). Statistical significance was calculated using the Mann-Whitney test (***: p<0.001). Scale bar: 1 μ m.

E. Super-resolution dSTROM imaging of immunocytochemistry on DIC21 primary cortical neurons using AUTS2 antibody (white dots) and conventional imaging of phalloïdine (green) and SYNPO (red). Merged image is shown. Proportion of dendritic spines displaying Auts2 cluster only inside the spine head (green) or the neck (salmon), in both neck and head (grey) or with no Auts2 clusters (blue) for dendritic spines with (SYNPO+) or without (SYNPO-) SYNPO. Scale bar: 1µm.



Figure 2: Morphological phenotypes of dendritic spines and decrease in spontaneous excitatory post-synaptic current (sEPSC) induced in *Auts2*-silenced cortical primary neurons

A. Dendritic spines from E17.5 primary cortical neurons transfected at DIC7 with sh-NS or sh1 and fixed at DIC21. Mushroom spines are indicated with arrows. Scale bars=10µm.

B. Dendritic spine length and spine width are calculated respectively as the length of the neck and the width of the synaptic button of the mushroom spines present on 75 μ m dendritic fragment samples. *p<0.01; **p<0.001; ***p<0.0001

C. Examples of whole-cell voltage-clamp recordings of sEPSC recorded in post-synaptic cortical neurons transfected either with sh-control (sh-NS) or sh-Auts2 (sh1).

D. Quantification of whole-cell voltage-clamp recordings sEPCS amplitudes and frequencies. Note that silencing of Auts2 induced a significant decrease in sEPSC amplitude without significant change in sEPSC frequency.

E. Quantal distribution of whole-cell voltage-clamp recordings sEPCS of control as compared to Auts2-silenced post-synaptic cortical neurons. An enlargement of the distribution of sEPSC for the lowest quantal release is inserted for both control and *Auts2*-silenced postsynaptic cortical neurons. Note that this quantal component amplitude decrease from - 9.7 to -6.25 pA suggesting a post-synaptic effect of Auts2 silencing.



Figure 3: Effect of Auts2 knockdown on Ttc3-Akt2 complexes and rescue of synaptic currents by AKT2 intracellular injection.

A-C: Quantification of TTC3-AKT2 complexes upon changes of Auts2 levels using *in situ* PLA in primary cortical neurons. *In situ* PLA was performed on primary cortical neurons fixed at DIC3 (red fluorescence) using anti-Ttc3 and anti-Akt antibodies. Scale bars=10µm.

A: Representative example of a neuron transfected with control sh-RNA [sh-NS] (green fluorescence), and of Ttc3-Akt2 complexes (red fluorescence) are shown in (A1). (A2) enlarged view of the merged images.

B: Representative example of a neuron transfected by sh1 (green fluorescence), and of Ttc3-Akt2 interactions (red fluorescence) are shown in (B1). (B2) enlarged view of the merged images.

C: Number of complexes under control [sh-NS] and Auts2 knockdown [sh1] conditions.

D-E Intracellular AKT2 injection in Auts2-depleted neurons rescues synaptic current alterations

D: Sample whole-cell voltage-clamp recordings of sEPSC recorded in post-synaptic cortical neurons following heat-denatured or native Akt2 injection.

E. Quantification of sEPCS amplitudes as a function of time following seal openings. Note the recovery of sEPCS 40 minutes after Akt2 injection.

Supplementary Material

Methods

Yeast Two-hybrid screen

Random primers were used to construct a cDNA library from human foetal brain mRNA (Invitrogen) into the pP6 plasmid derived from the original pACT2, using blunt end ligation of a Sfil linker. A 97 million independent clone library was obtained in E. coli. The DNA from this library was transformed into S. cerevisiae by classical lithium acetate protocol. Ten millions independent colonies were collected, pooled, and stored at -80°C as aliquot fractions of the same library. Two-hybrid screens using a cell-to-cell mating protocol was performed as previously described42. Full length Aust2 was cloned in lexA based pB27 bait vector and 50 millions interactions have been tested and selected using His3 reporter. Performed by Hybrigenics S.A., Paris, France (http://www.hybrigenics.com/services.html).

Probe synthesis and quantitative in situ hybridization (ISH)

We used AUTS2 human and Auts2 mouse cDNA clones from the RZPD Library (IRALp962I2117Q2 and IMAGp998A169209Q1 respectively). We synthesized [α 35S]-rUTP (800 Ci/mmol, Amersham) labeled riboprobe (human probe of 1171 bp linearized with BamHI and mouse probe of 705 bp linearized with EcoRI), using the P1460 riboprobe in vitro transcription system (Promega) according to the manufacturer's instructions. Paraffinembedded coronal 15 µm sections were hybridized with radiolabelled RNA probe (diluted to 105 cpm/µl in 50% formamide hybridisation buffer) in 50% formamide at 50°C. Sections were successively washed in 50% formamide, 2 x SSC, 10 mM DTT at 65°C and then in increasingly stringent SSC washing solution, with a final wash at 0.1 x SSC at 37°C. Hybridization was carried out with both antisense and sense riboprobes, in the conditions described in 1 and no signal were obtained with sense riboprobes. Expression was quantified with a Biospace Micro Imager, using Betavision analysis software (Biospace Instruments)[*1,2*].

Primary cell cultures, cell line culture and transfection

We used mice of the C57BL6 strain. E17.5 mouse telencephalic and hippocampal neurons were at the same time dissociated enzymatically (0.25% trypsin, DNase), mechanically triturated with a flamed Pasteur pipette, and plated on 35mm dishes (8x10⁵ cortical cells and 4x10⁵ hippocampal cells per dish) coated with poly-DL-ornithine (Sigma), in DMEM (Invitrogen) supplemented with 10% foetal bovine serum. Four hours after plating, DMEM was replaced by Neurobasal® medium (Invitrogen) supplemented with 2mM glutamine and 2% B27 (Invitrogen). Cells were transfected with constructs using Lipofectamine and Plus-Reagent (Invitrogen), as described by the manufacturer. Neurite and spine analysis included at least 12 cells per condition and was carried out with ImageJ software (Wayne Rasband, NIH). HeLa cell lines were cultured in DMEM supplemented with 10% of foetal bovine serum in 100mm dishes and were transfected close to confluence with Arrest-in (OpenBiosystems).

Constructs

To silence mouse Auts2, we used sh-RNA p-GIPZ vector (#RMM4431-98725112 for sh1 and #RMM4431-99010075 for sh2), as well as the control Non-Silencing sh-RNA (scrambled #RHS4346) from OpenBiosystems clone library (www.openbiosystems.com).

Immunocytochemistry and confocal microscopy

Cells were fixed by incubation in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, permeabilised by incubation for 10 min at room temperature in 0.3% Triton X-100 in PBS and blocked for 30 min at RT with 3% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS. The cells were then incubated overnight at 4°C with primary antibodies (rabbit polyclonal anti-PSD95, 1:500, Synaptic Systems; mouse

monoclonal anti-Synapsin1; 1:500, Synaptic Systems; chicken polyclonal anti-MAP2, 1:10,000, Abcam; mouse monoclonal anti-TAU1, 1:500, Chemicon; rabbit polyclonal anti-AUTS2 6361 and 6362, 1:50, generated by Eurogentec, mapping 1-15 aa and 187-197 aa respectively, that have been purified using the Melon Antibody Purification Kit (ThermoScientific) according to the instructions of the manufacturer), diluted in the blocking solution. Cells were washed three times within PBS and incubated for 1h at room temperature with the secondary antibody (Alexa-488 anti-mouse IgG, Alexa-488 anti-rabbit IgG, Alexa-555 antimouse IgG, Alexa-555 anti-rabbit IgG and Alexa-647 anti-chicken IgG, 1:1,000, Invitrogen) diluted in the blocking solution. Cells were washed three times solution. Cells were washed three times solution. Cells were washed three times using anti-rabbit IgG and Alexa-647 anti-chicken IgG, 1:1,000, Invitrogen) diluted in the blocking solution. Cells were washed three times solution. Cells were scanned using laser scanning confocal microscope (Carl Zeiss, LSM 510).

In situ proximity ligation assays (PLA)

Cells were fixed by incubation in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, cells were washed two times within PBS and PLA was performed according to the instructions of the manufacturer (DuoLink, Eurogentec). Primary antibodies used were rabbit polyclonal anti-AUTS2 6361 and 6362, 1:50, generated by Eurogentec, mapping 1-15 aa and 187-197 aa respectively, that have been purified using the Melon Antibody Purification Kit (Thermo Scientific) according to the instructions of the manufacturer); goat polyclonal anti-TTC3, 1:500, Santa-Cruz; rabbit polyclonal anti-AKT2, 1:500, Abcam; goat polyclonal anti-CITRON, 1:500, Santa-Cruz and goat polyclonal anti-TOM20, 1:500, Santa Cruz. Sections were scanned using laser scanning epifluorescence microscope (Carl Zeiss, AxioPlan2 Imaging).

Statistical analysis

T-tests were performed with Excel Software and KS-test (Kolmogorov-Smirnov) with web software (Kirkman, T.W. (1996) Statistics to Use. http://www.physics.csbsju.edu/stats/ (2011).

Protein extraction and Western blot analysis

HeLa cells transfected with shNS (n=5) and shD (n=5) were homogenised in RIPA buffer (0.5% NADOC, 1% NP40, 1% SDS 10% in PBS with 1x Protease Inhibitor Cocktail and 1% Phosphatase Inhibitor cocktails) for 10 min on ice and centrifuged 15 min at 10,000g at 4°C. The supernatants were stored at -80°C until used and lysate protein concentration was determined using the DCTM Protein assay (Biorad). Ten micrograms of protein was heated in the loading buffer and loaded on 5–12% gradient NuPage gels (Invitrogen). After the transfer on nitrocellulose membranes, filters were incubated for 1h at room temperature in blocking solution (5% non-fat dried milk and 0.1% Tween 20 in PBS) and then overnight at 4°C with the primary antibody (purified rabbit anti-Auts2 1:50, mouse anti- β -actin, 1:30,000, Sigma). Filters were washed in 0.1% Tween 20 PBS and incubated 1h at room temperature with the secondary antibody HRP-conjugated (anti-rabbit and anti-mouse IgG, 1:5,000, ECL). Membranes were washed within 0.1% Tween 20 PBS. Chemiluminescent detection reagents (Pierce) were used according to company instructions and membranes were exposed to autoradiography film (Kodak Biomax). Protein bands were then subjected to densitometric analysis using ImageJ software (Wayne Rasband, NIH).

Transcription factor sites analysis

Neandertal sequences were downloaded from "UCSC neandertal portal". We used neandertal contigs (mixed of 6 specimens sequenced). We selected fragments aligned on hg18 reference and analyzed only human sequences which had neandertal homologues. The analysis was conducted on sequences having exactly the same number of human and neandertal nucleotides. We obtained 12,437 pairs containing 1,212,584 nt for each species. We excluded from the analysis pairs which were identical between human and neandertal. This procedure reduced the dataset to 7,193 sequence pairs containing 797,226 nt (r1

254,125 nt, r2 241,463 nt, r3 301,638 nt) AUTS2 locus was analyzed with the Genomatix MatInspector program for transcription factor sites discovery with stringent conditions (core sim = 0.95 and opt +0.1) for both species. We counted only site predictions which were present in one lineage on sequence pair. For comparison we randomly selected aligned regions (200,000 pair containing 18,890,929 nt, with elimination of identical pairs it was reduced to 115,069 pairs containing 12,312,338 nt) across whole genome and did the same analysis. We used boostrap approach to infer significance of matrix family hits on AUTS2 locus. We randomly selected regions, counted the number of nucleotides of the region in the randomly selected regions, counted the number of matrix family hits and did this analysis 10,000 times. We could estimate the number of matrix family hits for region of approximately the same length of the region of interest and calculated z-score for each matrix family and by region.

Electrophysiological recordings of cortical neurons

7 to 10 days in vitro cortical neurons grown on coverslips in neurobasal medium supplemented with B27 were transfected either with sh-control (sh-NS) or sh-Auts2 (sh1) using lipofectamine (Invitrogen, France) according to manufacturer instructions. We used whole-cell patch-clamp recording of transfected neurons 48 to 72h after transfection. The cells were perfused voltage-clamped at -70 mV. The whole-cell with a Tyrode solution containing (in mM): 150 NaCl, 2 CaCl2, 1 MgCl2, 4 KCl, 10 glucose, 10 HEPES (pH 7.4). Spontaneous excitatory postsynaptic currents (sEPSCs) were studied in transfected cells voltage-clamped at -70mV. Patch electrodes, fabricated from thick borosilicate glass, were pulled and fire-polished to a final resistance of 3-4 M Ω and filled with the standard internal solution (in mM): 100 CsMES, 20 CsCl, 2 MgCl2, 5 ethylene glycol tetraacetic acid (EGTA), 10 HEPES, 4 ATP, and 15 phosphocreatine (pH 7.4). AKT2 protein (ab 79798) was obtained from Abcam.

References

 Charpak G, Dominik W, Zaganidis N. (1989) Optical imaging of the spatial distribution of beta-particles emerging from surfaces. Proc Natl Acad Sci USA 86,1741–1745(1989).
Lepagnol-Bestel AM et al. SLC25A12 expression is associated with neurite outgrowth and is upregulated in the prefrontal cortex of autistic subjects. Mol Psychiatry. 13, 385-397 (2008). Supplementary Table S1: AUTS2, psychiatric, neurodevelopmentaland neurological diseases

Genetic variations	Phenotype	Reference
Translocation breakpoint	ASDs	Sultana et al., 2002
Translocation breakpoint	ASDs	Huang et al., 2010
Chromosomal inversion	ASDs	Bakkagoglu et al., 2008
CNVs	ASDs	Cusco et al., 2009
Balanced translocation	Mental Retardation	Kalscheur et al., 2007
CNVs	ADHD	Elia et al., 2010
SNP (GWAS)	Bipolar	Hamshere et al., 2009
Modified DNA methylation	Schizophrenia & Bipolar	Mill et al., 2009
CNVs	Epilepsy	Mefford et al., 2010

Abbreviations:

ADHD (Attention-Deficit Hyperactivity Disorder); ASDs (Autism Spectrum Disorders); CNV (Copy Number Variation); SNP (Single nucleotide polymorphism); GWAS (Genome Wide Association Study).

Supplementary Table S2: Identification of statistically significant putative transcription factor sites in Human and Neandertal AUTS2 loci.

TF matrix name	n	Gene name	Entrez Neuronal annotation	Zscore
IKRS	3	IKZF1	Forebrain development	3.30
PLZF	1	ZBTB16	Central nervous system development	3.07
GREF	3	AR / PGR	Axon & dendrite / Neuron projection	4.27
PTF1	1	PTF1A	Neuron fate commitment	3.56
HESF	2	HES1	Nervous system development	3.07

TF matrix name	n	Gene name	Entrez Neuronal annotation	Zscore
GCNR	1	GCNF / NR6A1	Neurogenesis	7.84
AP4R	1	TFAP4	0	3.24
RP58	3	ZNF238	0	5.65
ZF35	1	ZNF35	0	3.51
CP2F	2	LSF / CP2 / SEF	0	5.03

We identified putative transcription factor (TF) sites using statistical approach based on 1,000 samples bootstrap in order to select sites with Zscores \geq 3 or \leq -3.

We used Entrez Gene database to identify genes having evidence of neuronal function (ie in summary or in process and component of Gene Ontology Annotation) as in Prabhakar et al., 2006.



Supplementary Figure S1: AUTS2 expression during human brain development & locus positive selection

Coronal sections of 8-week (A) and 22-week-old (B) human embryos (B1 anterior section and B2 posterior section) hybridized with AUTS2 antisense radioactive riboprobe.

di: diencephalon; ic: internal capsule; fr c: frontal cortex; ge: germinal zone; hip: hippocampus; in: insular cortex; mes: mesencaphalon; nc: nucleus caudate; nl: nucleus lenticular; rho: rhombencephalon; rl: rhombic lip; tc: temporal cortex; tel: telencephalon. Scale bar=1mm.

C. quantification indicating expression of AUTS2 both in cortex and hippocampus at 22week-old human embryonic development with a significantly higher expression level in cortical regions than in hippocampus. * = p<0.05

D. Diagram of the chromosome 7 locus including the AUTS2 gene. Region 2 (377,373bp) was identified as displaying a signature of positive selection in the human compared to Neandertal lineage13. Statistically significant (Zscores \geq 3 or \leq 3) putative transcription factor (TF) matrix sites using Genomatix suite are indicated for Human and Neandertal AUTS2 loci. The TF matrix family names for the Human and Neandertal sites are indicated.

E. Human-associated TF sites are disproportionately associated with neuronal expression using Entrez Gene neuronal annotations (p=2.51x10-4; Fisher's exact test).



Supplementary Figure S2: Nuclear and dendritic localization of *Auts2* in primary cortical neurons

Immunocytochemistry on cortical neurons fixed at DIC21, using DAPI to stain the nucleus (A1-B1), 6361-Auts2 antibody (A2-B2) and anti-Synapsin1 (A3-B3). Merged images are shown in (A4-B4). Scale bar=10µm (A); Scale bar=5µm (B).



Supplementary Figure S3: Dendritic and dendritic spine localisation of mouse *Auts2* protein in primary cortical neurons

Immunocytochemistry on DIC21 primary cortical neurons using anti-MAP2 (A1) to visualize dendrites, anti-synapsin1 as presynaptic marker (B1), anti-PSD95 as postsynaptic marker

(C1) and 6361-Auts2 antibody (A2-B2-C2). Merged image is shown in (A3). Scale bars=10 μ m.



Supplementary Figure S4.

Dendritic spine localization of mouse *Auts2* protein and protein-protein interaction analysis using Yeast-two-hybrid and *in situ* proximity ligation assays in primary cortical neurons

A. Immunocytochemistry on DIC21 primary cortical neurons using 6361-Auts2 antibody (red fluorescence) and the presynaptic marker Synapsin1 (green fluorescence upper panel) or the postsynaptic marker PSD95 (green fluorescence lower panel). Pearson's coefficient Rr = $56.5\% \pm 5.1\%$, Rr = $80.6\% \pm 1.9\%$, respectively; n=11 and n=7. (Scale bars=10µm).

- B. Yeast two-hybrid one-by-one assays revealed TTC3 as an interactor of AUTS2.
- C+: interaction positive control
- C- (pB27ø + pP7ø): empty pB27 vector + empty pP7 vector
- C- (AUST2 + pP7ø): pB27-AUST2 + empty pP7 vector
- C- (pB27ø + TTC3): empty pB27 vector + pP6-TTC3
- AUST2 + TTC3: pB27-AUST2 + pP6-TTC3

C. Schematic representation of the AUTS2, TTC3, AKT and CITRON interactions. In red are the interactions demonstrated in the study.

D. *In situ* proximity ligation assays PLA on primary cortical neurons fixed at DIC19 (red fluorescence) using 6362-Auts2 and anti-Ttc3 antibodies (D1), anti-Ttc3 and anti-Akt antibodies (D2) as a positive control, and anti-Akt and anti-Citron antibodies (D3). Immunohistochemistry was performed using anti-MAP2 (green fluorescence) to visualize dendrites and Hoechst (blue fluorescence) to visualize nucleus. Enlargements of the merged images are shown in lower panel. Scale bars=10µm.

E. Mean interaction point number calculated in dendrites of at least 11 cortical neurons at DIC19. *In situ* PLA was used to analyze the interaction between Auts2 and Ttc3, between Ttc3 and Akt2 as a positive control, and between Akt2 and Citron. As a negative control we analyzed interaction between Tom20 and Auts2. * p < 0.05; ** p < 0.005 and *** p < 0.0005.

Supplementary Figure S5: In situ proximity ligation assay validation

In situ proximity ligation assays (PLA) on primary cortical neurons fixed at DIC19 (red fluorescence) using 6362-Auts2 and anti-Tom20 antibodies as a negative control. Immunocytochemistry was performed using anti-MAP2 (green fluorescence) to visualize dendrites and Hoechst (blue fluorescence) to visualize nucleus. Enlargements of the merged images are shown in lower panel. Sale bars=10µm.



Supplementary Figure S6: AUTS2, CITRON and TTC3 mRNA levels in the human central nervous system during early development and at mid-gestation

A. Sagittal sections of 8-week-old human embryos hybridized with AUTS2 (A1), TTC3 (A2) and CITRON (A3) antisense radioactive riboprobes. The three transcripts were detected in telencephalon (tel), ganglionic eminence (ge), hippocampus anlagen (hipp), cerebellum anlagen (Cb) and liver (li) but not in heart (h) and lung (lu).

B-C. Coronal sections of 15-week-old human brains hybridized with AUTS2 (B1-C1), TTC3 (B2-C2) and CITRON (B3-C3) antisense radioactive riboprobes. The three transcripts were detected in frontal cortex (fr c), tempral cortex (temp c), insular cortex (in) and germinal zone (ge) but nor in nucleus caudate (nc), nucleus lenticular (nl) and internal capsule (ic).

D. Coronal sections of 19-week-old human brains hybridized with AUTS2 (D1), TTC3 (D2) and CITRON (D3) antisense radioactive riboprobes. The three transcripts were detected in frontal superior cortex (sup fr), lateral frontal cortex (lat fr), insular cortex (ins), galglionic eminence (ge), internal capsule (ic) and nucleus caudate (nc) but not in thalamus (th), putamen (p) and lateral temporal cortex (lat temp). Scale bars=1mm.



Supplementary Figure S7: Validation of the Auts2 shRNA vectors

Immunoblot quantification of Auts2 protein in HeLa cells transfected at DIC2 with shNS or sh1 and harvested 48 hours later (n=5). Expression was normalized against the β -actin protein. Independent samples were loaded from sh-NS (lanes 1-2) or sh1 (lanes 3-4) transfected HeLa cells. *p<0.01.



Supplementary Figure S8: Cellular phenotypes in *Auts2*-silenced cortical primary neurons

A-B. Axonal and dendritic complexity analysis on cortical neurons transfected at DIC1 with shNS, sh1 or sh2 and fixed at DIC2 or transfected at DIC2 and fixed at DIC3, DIC4 and DIC5. Evaluation of axons and dendrites was performed taking into account the axonal (A1) or dendritic (B1) length and axonal (A2) or dendritic (B2) branching point number. *p<0.01; **p<0.001; ***p<0.0001

C. Quantification of dendritic spine density in cortical neurons transfected at DIC7 with shNS or sh1 and fixed at DIC21. Total dendritic spine number was calculated per 75 μ m dendritic fragments (C1) and classified by spine morphotype: mushroom, stubby and filopodia (C2).



Supplementary Figure S9: Cellular phenotypes in *Auts2*-silenced hippocampal primary neurons

A-B. Axonal and dendritic complexity analyses on hippocampal neurons transfected at DIC2 with shNS, sh1 or sh2 and fixed at DIC3, DIC4 and DIC5. Evaluation of axons and dendrites was performed taking into account the axonal (A1) or dendritic (B1) length and axonal (A2) or dendritic (B2) branching point number. *p<0.01; **p<0.001; ***p<0.0001



Titre : Quantification du transport intraneuronal par suivi de nanodiamants fluorescents. Application à l'étude de l'impact fonctionnel de facteurs de risque génétiques associés aux maladies neuropsychiatriques.

Mots clés : nanodiamant fluorescent, suivi de particule unique, transport intraneuronal, maladie mentale, facteur de risque génétique.

Résumé : L'identification de biomarqueurs des maladies mentales telles que l'autisme, la schizophrénie ou la maladie d'Alzheimer, est d'une importance capitale non seulement pour établir un diagnostic objectif, mais aussi pour suivre l'effet des traitements. La création et le maintien de fonctions neuronales sub-cellulaires, telle que la plasticité synaptique, sont fortement dépendants du transport intraneuronal, essentiel pour acheminer d'importants composants à des positions spécifiques. Un transport actif défaillant semble être partiellement responsable d'anomalies de la plasticité synaptique et de la morphologie neuronale présentes dans de nombreuses maladies neuropsychiatriques. Cette thèse décrit (i) la mise au point d'une méthode de quantification du transport intraneuronal reposant sur le suivi de nanoparticules de diamants fluorescents (fNDs); (ii) l'application de cette technique simple et faiblement invasive à l'analyse fonctionnelle de variants génétiques associés à des maladies mentales.

Ce manuscrit comporte quatre chapitres. Le premier détaille l'architecture polygénique complexe des maladies mentales

et démontre la pertinence d'étudier le transport intraneuronal. Les deuxième et troisième chapitres sont dédiés à la méthode et détaillent les stratégies d'internalisation des fNDs, les outils de quantification du transport intraneuronal et la validation de la technique. La forte brillance, la photostabilité parfaite et l'absence de toxicité cellulaire font des fNDs un outil de choix pour étudier la dynamique du transport intraneuronal sur une durée d'observation de plusieurs heures avec une haute résolution spatiotemporelle et une bonne puissance statistique. Enfin, dans le quatrième chapitre, nous appliquons cette nouvelle méthode d'analyse fonctionnelle pour étudier l'effet de variants génétiques associés à l'autisme et à la schizophrénie. Pour cela, nous utilisons des lignées de souris transgéniques ayant une faible surexpression des gènes MARK1 et SLC25A12, ainsi que des AAV-shRNA pour induire une haplo-insuffisance du gène AUTS2. Notre méthode de diagnostic moléculaire s'avère suffisamment sensible pour déceler des variations fines de la dynamique du transport intraneuronal, ouvrant la voie à de futurs développements en nanomédecine translationnelle.

Title : Quantification of intraneuronal transport by fluorescent nanodiamond tracking. Application to the screening of the functional impact of neuropsychiatric disease-related genetic risk factors.

Keywords : Fluorescent nanodiamond, single-particle tracking, intraneuronal transport, brain disease, genetic risk factor

Abstract : The identification of molecular biomarkers of brain diseases as diverse as autism, schizophrenia and Alzheimer's disease, is of crucial importance not only for an objective diagnosis but also to monitor response to treatments. The establishment and maintenance of subcellular neuronal functions, such as synaptic plasticity, are highly dependent on intracellular transport, which is essential to deliver important materials to specific locations. Abnormalities in such active transport are thought to be partly responsible for synaptic plasticity and neuronal morphology impairment found in many neuropsychiatric and neurodegenerative diseases. This thesis reports (i) the development of a quantification technic of intraneuronal transport based on fluorescent nanodiamonds (fNDs) tracking; (ii) the application of this simple and minimally invasive approach to the functional analysis of neuropsychiatric disease-related genetic variants.

This manuscript falls into four chapters. The first one details the complex polygenic architecture of mental disorders and demonstrates the disease relevance of monitoring the intraneuronal transport. The second and the third chapters are dedicated to the nanodiamond-tracking assay and describe the fNDs internalisation strategies, the spatiotemporal quantitative readouts and the validation of the technique. The high brightness, the perfect photostability and the absence of cytotoxicity make fNDs a tool of choice to perform high throughput long-term bioimaging at high spatiotemporal resolution. Finally, in the fourth chapter, we apply this new functional analysis method to study the effect of genetic variants associated to autism and schizophrenia. We established transgenic mouse lines in which MARK1 and SLC25A12 genes were slightly overexpressed, and AAVshRNA to induce AUTS2 gene haploinsufficiency. Our molecular diagnosis assay proves sufficiently sensitive to detect fine changes in intraneuronal transport dynamic, paving the way for future development in translational nanomedicine.

