GENETIC, EPIGENETIC AND TRANSCRIPTOME STUDIES OF TOURETTE SYNDROME AND TIC DISORDERS

PhD thesis

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1. ABBREVIATIONS

3′UTR: 3′ Untranslated Region

5-hmC: 5-hydroxymethyl-cytosine

6-OHDA: 6-hydroxydopamine

AADAC: Arylacetamide deacetylase

ACP1: Acid phosphatase 1, soluble

ADAM1: ADAM metallopeptidase domain 1A (pseudogene)

ADHD: Attention Deficit Hyperactivity Disorder

ADORA1: Adenosine A1 receptor

ADORA2A: Adenosine A2a receptor

AIM: Abnormal Involuntary Movement

ARC: Activity regulated cytoskeleton associated protein

ATF3: Activating transcription factor 3

BAG3: BCL2 associated athanogene 3

BLM: BLM RecQ like helicase

Bp: Base Pair

BRD1: Bromodomain containing 1

BRPF3: Bromodomain and PHD finger containing 3

BTBD9: BTB (POZ) domain containing 9

CCND3: Cyclin D3

CDC37: Cell division cycle 37

CDH13: Cadherin 13

CDH2: Cadherin 2, type 1, N-cadherin neuronal

CDK19: Cyclin dependent kinase 19

CDKN1A: Cyclin dependent kinase inhibitor 1A

CHAT: Choline acetyltransferase

CHRNA7: Cholinergic receptor, nicotinic, alpha 7 (neuronal)

CLINT1: Clathrin interactor 1

CNR1: Cannabinoid receptor 1

CNTN6: Contactin-6

CNTNAP2: Contactin associated protein-like 2

CNV: Copy Number Variation

COL27A1: Collagen, type XXVII, alpha 1

COL8A1: Collagen, type VIII, alpha 1

COMMD1: COMM domain-containing protein 1

COMT: Catechol-O-methyltransferase

CpG: Cytosine-Phosphate-Guanine dinucleotide

CREB1: cAMP-responsive element binding protein 1

CREM: cAMP responsive element modulator

CRH: Corticotropin releasing hormone

CSRNP1: Cysteine and serine rich nuclear protein 1

CSTC: Cortico-Striato-Thalamo-Cortical

DA: Dopamine

DMS: Differentially Methylated Site:

DNMTs: DNA Methyltransferases

DPP6: Dipeptidyl-peptidase 6

DRD1: Dopamine receptor D1

DRD2: Dopamine receptor D2

DRD4: Dopamine receptor D4

DSM-5: Diagnostic and Statistical Manual of Mental Disorders

DUSP14: Dual specificity phosphatase 14

EGR4: Early growth response 4

EHD4: EH domain containing 4

ESR: Early Stage Researcher

EWAS: Epigenome-Wide Association Study

FGF13: Fibroblast growth factor 13

FOS: Fos proto-oncogene, AP-1 transcription factor subunit

FOSB: FosB proto-oncogene, AP-1 transcription factor subunit

FRMD6: FERM domain containing 6

GAA: Glucosidase alpha, acid

GABBR1: Gamma-aminobutyric acid type B receptor subunit 1

GADD45B: Growth arrest and DNA damage inducible beta

GADD45G: Growth arrest and DNA damage inducible gamma

GDNF: Glial cell Derived Neurotrophic Factor

GGRI: GWAS Replication Initiative

Gpe: Globus Pallidus Externus

Gpi: Globus Pallidus Internus

GPR3: G protein-coupled receptor 3

GRIK3: Glutamate ionotropic receptor kainate type subunit 3

GRIK4: Glutamate ionotropic receptor kainate type subunit 4

GRIN2B: Glutamate ionotropic receptor NMDA type subunit 2B

GRM4: Glutamate metabotropic receptor 4

GRM8: Glutamate metabotropic receptor 8

GSTP1: Glutathione s-transferase pi 1

GWAS: Genome Wide Association Study

HDC: Histidine decarboxylase

hEAE6: Homolog of Esa1-associated factor 6

HIST1H4E: Histone cluster 1 H4 family member e

HPLC-ECD: Electrochemical detection technique coupled with high-performance liquid chromatography

HSPA5: Heat shock protein family A (Hsp70) member 5

HTR2A: 5-hydroxytryptamine Receptor 2A

ID1: Inhibitor of DNA binding 1, HLH protein

IGSF9B: Immunoglobulin superfamily member 9B

Illumina 450K: Infinium HumanMethylation450 Bead Chip Kit

ILR1N: Interleukin 1 receptor antagonist

IMMP2L: Inner mitochondrial membrane peptidase subunit 2

ING5: Inhibitor of growth 5

INHBA: Inhibin beta A subunit

IRS2: Insulin receptor substrate 2

JUN: Jun proto-oncogene, AP-1 transcription factor subunit

JUNB: JunB proto-oncogene, AP-1 transcription factor subunit

KCNK9: Potassium channel two pore domain subfamily K member 9

KLF4: Kruppel like factor 4

LHX6: LIM homeobox 6

LMO1: LIM domain only 1

MAOA: Monoamine oxidase A

MCPerm: Monte Carlo Permutation

MECP2: Methyl-CpG-binding protein 2

MEIS1: Meis homeobox 1

MIDN: Midnolin

MORN4: MORN repeat containing 4

NDUFV1: NADH ubiquinone oxidoreductase core subunit V1

NFIL3: Nuclear factor, interleukin 3 regulated

NPAS4: Neuronal PAS domain protein 4

NPTX2: Neuronal pentraxin 2

NPY: Neuropeptide Y

NR4A1: Nuclear receptor subfamily 4 group A member 1

NR4A3: Nuclear receptor subfamily 4 group A member 3

NRGN: Neurogranin

NRXN1: Neurexin 1

NTN4: Netrin 4

OCD: Obsessive-Compulsive Disorder

OLFM1: Olfactomedin1

OR: Odds Ratio

Ov.T: Over-Transmitted

P: P-value

PANDAS: Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal infection

PARP1: Poly (ADP-ribose) polymerase 1

PD: Parkinson's disease

PDE10A: Phosphodiesterase 10A

PDXK: Pyridoxal kinase

PDYN: Prodynorphin

PER1: Period circadian clock 1

PIGX: Phosphatidylinositol glycan anchor biosynthesis class X

PIM3: Pim-3 proto-oncogene, serine/threonine kinase

PLAT: Plasminogen activator, tissue type

PLEKHG3: Pleckstrin homology and RhoGEF domain containing G3

PTGS2: Prostaglandin-endoperoxide synthase 2

PVR: Poliovirus receptor

RELN: Reelin RELN

REM2: RRAD and GEM like GTPase 2

RHEB: Ras homolog enriched in brain

SCG2: Secretogranin II

SEMA7A: Semaphorin 7A (John Milton Hagen blood group)

SEMAG4G: Semaphorin-4G

SERT: Serotonin transporter

SERTAD1: SERTA domain containing 1

SGK1: Serum/glucocorticoid regulated kinase 1

SH3KBP1: SH3 domain containing kinase binding protein 1

SIK1: Salt inducible kinase 1

SLC25A48: SLC25A48 solute carrier family 25 member 48

SLC32A1: Solute carrier family 32 member 1

SLC6A3: Solute carrier family 6 member 3

SLC6A4: Solute carrier family 6 (neurotransmitter transporter) member 4

SLITRK1: SLIT and NTRK like family member 1

SN: Substantia Nigra

SNP: Single Nucleotide Polymorphism

SNTG1: Syntrophin gamma 1

SRXN1: Sulfiredoxin 1

STAT3: Signal transducer and activator of transcription 3

STN: Subthalamic Nucleus

TAC1: Tachykinin precursor 1

TDT: Transmission Disequilibrium Test

TNF: Tumor necrosis factor

TPH2: Tryptophan hydroxylase 2

TS: Tourette Syndrome

TSGeneSEE: The Tourette Syndrome Genetics - Southern and Eastern Europe Initiative

VEGFA: Vascular endothelial growth factor A

VNTR: Variable Number of Tandem Repeat

Vs: Versus

WFS1: Wolframin ER transmembrane glycoprotein

XRCC1: X-ray repair complementing defective repair 1

2. INTRODUCTION

2.1 Tourette Syndrome

Gilles de la Tourette Syndrome (TS) is a neurodevelopmental disorder characterized by the appearance of a vocal and multiple motor tics (DSM-5) [1]. To be diagnosed with TS, considered a rare disease affecting about 1% of the world population, the symptoms need to manifest for at least 1 year. It usually lasts only during childhood and adolescence and it affects most the boys than girls (4:1). The probability of transmitting the disorder to the next generation is between 0.58 and 077 [2, 3] with a very high risk of inheritance in case of a first-degree relative (father-mother). To complicate the situation, TS rarely manifests alone: in 45-60% of the cases it goes along with obsessive-compulsive disorder (OCD) while in 60% of the cases it appears with attention deficit hyperactivity disorder (ADHD). OCD is one of the main disorders shared by TS patients. It is characterized by the presence of unwanted repetitive thoughts and behaviors [4]. Similar to Tourette, neuroimaging studies implicated the involvement of the cortico-striato-thalamo-cortical (CSTC) circuit in the pathophysiology (see below). Not surprisingly, genetic studies are pointing towards the involvement of serotonergic, glutamatergic and dopaminergic pathways, which are the same neurotransmitter implicated in TS (see below). ADHD, the other principal disorder shared by TS patients, usually refers to different form of behavioral impairment that affect the quality of life of the children. The most common symptoms are the manifestation of hyperactivity, inattention, impulsivity and issues in establishing social interactions. The genetics of ADHD is extremely complex and as for TS there are not many significant findings at genome wide levels, although it is believed that the dopaminergic pathway is involved in the etiology of ADHD [5]. Other types of behavioral disorders, such as autism and learning disabilities are also usual comorbidities in individuals with TS. In order to gain more information on such a rare disease, usually less funded than the study of major diseases, the European Union established the Marie Curie Initial Training Network (TS-EUROTRAIN 2012-2016) [6]. This network was composed of 10 research centers spread across Europe employing 12 PhD students spread across Europe aiming to study TS from different angles (bioinformatics, animal model, genetic,

neuroimaging) and to combine all the new discoveries to have a clearer picture of the pathology (see below).

2.1.1 TS Diagnosis

DSM-5 provides the following guidelines that allow a person to be diagnosed with TS:

- manifestation of at least two motor tics and at least one vocal tic
- manifestation of tics for at least one year
- manifestation of tics before the age of 18
- manifestation of tics not related to the intake of any medicine/drug
- manifestation of tics not related to other medical conditions

2.1.2 Definition of tics

A tic is usually defined as the appearance of an involuntary locomotor activity or vocal sound that manifests occasionally for a short amount of time with different frequency, intensity, duration or anatomical localization. As depicted in Table 1 a tic might be either vocal or motor, simple or complex [1].

Table 1: Classification of Tics

	Motor	Vocal
Single	blinking rolling the eye to twitch the nose	sniffing coughing snorting
Complex	squatting touching jumping	barking mimicking animal sounds yelling strings of words

In general, tics are more prone to manifest if the patient is in a situation of emotional distress, while they are reduced when the patient is focused/involved in daily activities [7]. The patient can usually feel when the tic is coming, a phenomenon called "premonitory urge", and after the tic is released the patient experiences a sense of relief. The symptoms usually manifest during childhood and disappear at the end of adolescence following the typical waxing and waning pattern that characterizes Tourette Syndrome [8, 9]. In a few cases, the symptoms also persist into adulthood. In some cases, the tics are subtle enough so that they do not affect too much the daily life of the patients, since they learnt very well how to control and live with them. However, in some cases, when tics are complex and severe, involving several body regions and loud vocalizations and the episodes last longer, they can be severely debilitating for the patients.

2.1.3 The Cortico-Striato-Thalamo-Cortical Circuit

The full neurobiological background of TS is not yet understood, but many scientific studies detected morphological and functional changes in the cortico-striato-thalamo-cortical (CSTC) circuit [10-12]. The CSTC circuit (Fig. 1) is formed by the interaction between pre-motor and motor cortices, striatum, globus pallidus internus (GPi), globus pallidus externus (GPe), subthalamic nucleus (STN), thalamus, and substantia nigra (SN). The physiological activation of this circuit usually results in the manifestation of voluntary movements as well as the repression of involuntary movements. For a movement to manifest, the motor cortex needs to be activated by the thalamus, which is usually inhibited by the STN-GPe-GPi. The activation of the direct pathway starts after the release of glutamate leading to the activation of striatal projection to substantia niagra (SN) and globus pallidus interna (GPi). These GABAergic cells, which have an inhibitory action, block the SN. The inhibition of SN results in the activation of the thalamic glutamatergic neurons. The subsequent activation of the cortex leads to locomotor movements [13, 14]. An abnormal activity of the basal ganglia might lead to manifestation of tics as a consequence of the dysregulation of thalamic activity, as well as higher firing of the motor cortex [15, 16].

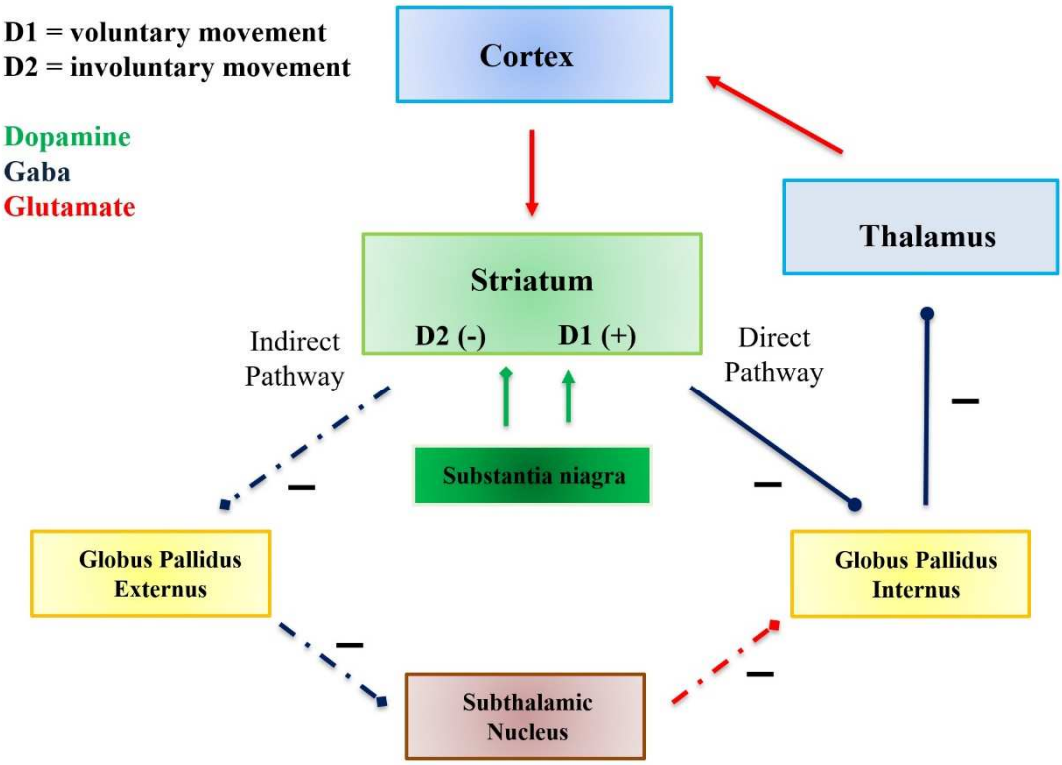

Fig. 1: Schematic diagram of the Cortico-Striato-Thalamo-Cortical (CSCT) circuit. The figure shows the area of the brain involved in the pathways that modulate both voluntary and involuntary movements. The green arrows indicate dopaminergic inputs, blue arrows indicate GABAergic inputs while the red arrows indicate glutamatergic inputs.

2.1.4 The hidden heritability of TS

Different studies analyzed the overall genetic contribution in the development of TS. A meta-analysis performed using all the human twin studies established that the heritability of tics/tic disorders is 0.45 [17]. Two other genetic studies assessed that the heritability of TS and tic disorders is 0.58 and 0.77, respectively [2, 3] with an elevated risk for first-degree relatives (father-mother). Heritability is a parameter that may help to understand the genetic architecture of complex disorders, and it usually refers to the proportion of the phenotypic abnormalities observed as a consequence of genetic variations [18]. The genetic variations identified in the association studies of Tourette Syndrome have been hinder by lack of statistical power, small sample sizes and clinical heterogeneity. It is

very unlikely that one single variation might cause all the variety of symptoms that appear in the patients. So, the genetic variations alone do not account for the overall TS heritability. Several other components might play a role. It is a phenomenon called "missed heritability" that might be explained by the contribution of environmental factors as well as epigenetic mechanisms in the development of TS (see below).

2.2 Genetic background of Tourette Syndrome

The following chapter describes some of the positive (but not always significant) genetic findings related to TS classified based on the extent of the DNA alteration:

- copy number variations (CNVs)
- variable number of tandem repeats (VNTRs)
- single nucleotide polymorphisms (SNPs)

Extensive literature research has been performed during this PhD project to identify potential candidate genes for genetic studies and it also resulted in the publication of a review on the genetic and epigenetic background of Tourette Syndrome [19]. It has been an exhaustive research, but it definitely does not include all the genetic studies ever published on TS. In the sections below, I am not going to described all the multiple and single positive studies involved in the literature research, because it is not the aim of this PhD thesis. I am going to provide a comprehensive genetic background that may help the reader to understand the reasoning behind the genetic and biological studies I performed during my PhD. More details about the genetic background of TS can be found in the published review [19].

2.2.1 CNVs and Tourette Syndrome

CNVs are characterized by rearrangements of a relatively large genomic region in an individual. It usually involves a deletion or duplication of a chromosomal segment that may alter the biological function of genes contained within and lead to the development of a disease.

The most studied gene with regards to CNV and Tourette Syndrome is the inner mitochondrial membrane peptidase subunit 2 (*IMMP2L*). This gene, located on chromosome 7q31, encodes for a protein involved in handling the signal peptide sequences used to direct mitochondrial proteins to the mitochondria. It was first discovered in a large TS family that had a balanced translocation [20]. *IMMP2L* was later identified in a male patient that had a de novo duplication [21]. It was also found disrupted in a male patient with a breakpoint on 7q31.1 [22]. Most recently, a CNV study in a Danish cohort of 188 TS patients found intragenic deletion of *IMMP2L* in seven patients [23].

In the last years many other CNVs have been associated to TS. One of the most robust study reported 8 patients with deletions of arylacetamide deacetylase (*AADAC*). This deletion was further investigated by meta-analysis in a large European sample of over 1000 TS patients and 100,000 controls that confirmed the role of *AADAC* deletions in TS pathogenesis [24].

The same lab [25] also reported the case of a patient with TS, OCD and ADHD carrying a de novo translocation leading to a truncation of the olfactomedin1 (*OLFM1*) gene. *OLFM1* is highly expressed in hippocampus and cerebral cortex and might have a role in a proper neuronal functioning [26].

One study focusing on large genomic rearrangements discovered a large duplication (600kb) of collagen type VIII alpha 1 chain (*COL8A1*) in 4 TS patients and a big deletion (400kb) of neurexin 1 (*NRXN1*) in 2 TS patients [27]. *COL8A1* plays structural role and contribute to organization, shape and mechanical properties of tissues and it has been linked to neural development throughout axonal guidance and synaptogenesis [28], while *NRXN1* mediates cell-cell interactions in the central nervous system and it has been associated to autism and schizophrenia [29, 30]. The involvement of exonic *NRXN1* deletions with the development of TS has been previously reported a Danish TS cohort [31] and also been recently proposed by a new study focused on the genomic burden of rare CNVs that showed how deletions in *NRXN1* have empirical thresholds for genome-wide significance [32].

The same study observed empirical genome-wide thresholds also for duplication involving contactin 6 (*CNTN6*), a novel association for TS [32].

A very interesting gene has been linked to TS via CNV, a study discovered a TS family carrying a microdeletion in the first exon of dipeptidyl peptidase like 6 (*DPP6*) [33]. This gene may be promising because it is expressed in the striatum, an area of the central nervous system with a clear role in the pathology of TS [34]. *DPP6* also influences dopamine levels and the dopaminergic pathway which is considered one of the major signaling cascade in TS.
A final example is the case of micro-duplications in cholinergic receptor nicotinic alpha 7 subunit (*CHRNA7*), a gene involved in fast signal transmission at synapses that is a major component of the brain nicotinic receptors, identified in 2 TS patients [35].

2.2.2 VNTRs and Tourette Syndrome

VNTRs are usually defined as short sequences of nucleotides, usually up to 100 base pairs (bp), organized in multiple copies grouped together and oriented in the same direction. VNTRs are divided into microsatellites (1 to 6 bp) or mini-satellites (14 to 100 bp) based on the length of the repeated units. The presence of a VNTR within a gene or nearby the regulatory region of genes, might affect gene expression or the protein structure, thus leading to development of diseases.
One of the most studied VNTR in Tourette Syndrome is linked to dopamine receptor D4 (*DRD4*). *DRD4* is a thoroughly studied gene in neuropsychiatric disorders, due to its pivotal role in regulating different brain functions such as the regulation of emotion, cognition and the modulation of neuroendocrine secretion [36]. This gene, located on chromosome 11p15.5, encodes for a G-protein coupled receptor, which inhibits adenylyl cyclase and it is known for encompassing a polymorphism consisting of a 48 bp VNTR [37, 38]. The most common VNTRs are the seven repeat units (a longer form of the gene), the four repeat units, and the two repeat units (a shorter form of the gene). It has been reported that the transmission of the long form of the gene (7 repeats) is over-transmitted in TS patients [39]. The same over-transmission of this VNTR has been subsequently observed in a different cohort of French-Canadian TS patients [40]. More recently, a family-based study on the 2x and 4x repeats revealed a significant transmission disequilibrium for both repeat-alleles [41].The authors speculated that the 2x repeat allele might play a protective role while the 4x repeat might confers risk.

The solute carrier family 6 member 3 (*SLC6A3*), also called *DAT1*, is another gene that has been associated with TS via VNTR. Coding for the dopamine transporter, it is a key component in the modulation of dopamine neurotransmission at the post-synaptic level. A genetic study showed that a 40 bp VNTR located in the 3′ untranslated region (3'UTR) of this gene is significantly associated with TS [42] and with increased tic manifestation [43].

The study of monoamine oxidase A (*MAOA*) also led to promising results even though there have not been new reports since 2004. This gene is involved in the breakdown and inactivation of monoamine neurotransmitters, such as serotonin and dopamine. The presence of a VNTR in exon 1 was found to be significantly higher in TS patients compared to controls [44]. In a second study it has been observed that a VNTR in the promoter region is also associated with TS [40]. It is important to note that *MAOA* is located on chromosome X and since TS has a male-female ratio of four to one, genetic studies need to correct for it thus affecting statistical power.

2.2.3 SNPs and Tourette Syndrome

Single nucleotide polymorphisms (SNPs) are the most common types of genetic variation among people. It represents a variation in the DNA sequence that occurs at a frequency higher than 1% in which a single nucleotide of the genome differs between members of a biological species or paired chromosomes [45]. In every human genome there are millions of SNPs but most of them are neutral and do not have any biological repercussion. However, there are some that may alter gene expression or cause structural changes in the encoded proteins thus leading to diseases.

Most of the TS genetic findings associated are conflicting since a lot of positive results have not been replicated in independent cohorts. Moreover, many genetic studies are done by individual labs with either small or medium sample size, which cannot easily reach statistical significance in a such complex genetic disorder. So below I will list these findings separately, according to their assumed predictive value.

2.2.3.1 Strong Positive Findings

One the most robust finding is represented by the top signal of the first genome wide association study (GWAS) of TS [46]. In a sample of 1285 cases and 4964 ancestry-matched controls, the SNP coded by rs7868992 was identified as the top hit (p = 1.85E-6). It is located in an intronic region of collagen, type XXVII, alpha 1 (*COL27A1*). The same SNP was associated to TS also in another study comprising of 260 Chinese trios [47]. The transmission disequilibrium test (TDT) showed the preferential transmission of the rs7868992 as well as rs4979357, however these results did not survive correction for multiple testing [47].

Some of the top hits (n = 42) of the first GWAS have been subsequently followed up [48]. The meta-analysis identified rs2060546 on netrin 4 (*NTN4*) as the strongest signal (p=5.8E-7). This gene might play an important role in the development of the nervous system by promoting axon outgrowth and guidance. The meta-analysis observed a similar trend (susceptibility risk for TS) for 26 of the 42 SNPs investigated, thus corroborating the reliability of the GWAS findings. More recently, a study comprising of 240 TS patients (Danish cohort) and 1006 healthy controls attempted to replicate the same *NTN4* finding [49]. The analysis did not show any significant association but the subsequent meta-analysis that combined samples from the TS GWAS Replication Initiative (GGRI) and the first TS-GWAS yielded a significant signal (OR = 3.74, p = 0.00018).

Before the publication of the first GWAS on Tourette Syndrome, the SLIT and NTRK like family member 1 (*SLITRK1*) gene was the most extensively studied. It regulates the growth of neurites during embryonic and postnatal development of the cortex, thalamus and the basal ganglia which are pivotal neuroanatomical area involved in TS pathology [50]. The first *SLITK1* study, in 2005, identified a rare mutation (var321) in the 3′UTR of the gene, an area with a putative binding site for microRNA-189 [51]. Since the original discovery, several studies in various populations have attempted to replicate the association of *SLITRK1* with TS but the results have been quite controversial. Indeed, many genetic studies failed to provided new positive findings as well as replicating the previous ones [52-57]. On the other hand, several genetic studies were able to provide support on the role of *SLITRK1* in the development of TS [58-62].

For instance, a family study performed on Canadian population detected a significant association between rs9593835 and TS [62]. The same SNP (rs9593835) has been positively replicated in a study performed on a Japanese population that found also an association of rs9546538 and rs9531520 with TS [60]. One more study performed on a large European population was also able to confirm the association between rs9546538 and rs9593835 with TS [61]. Moreover, a targeted resequencing approach has identified four variants that confirm the involvement of *SLITRK1* in the pathology [58].

One more largely investigated gene is the histidine decarboxylase (*HDC*). It was first discovered in a unique family where the father and all the affected off-springs had a variation that was causing a premature termination codon [59]. It was later on studied in a Chinese Han population that revealed no positive findings [63]. Lastly, a TDT test performed on 520 European detected an over-transmission of rs854150 and rs1894236 to be over-transmitted in patients [64], while a targeted resequencing approach identified a new deleterious variation that might increase TS risk [58]. Moreover, a *HDC* knockout mouse has also been proposed as a genetic model of TS [65, 66].

The last example of a very deeply investigated gene is BTB (POZ) domain containing 9 (*BTBD9*), which has been widely analyzed in TS leading once again to controversial results. The dispute is centered around rs4714156, rs9296249, and rs935727: one study on Canadian population showed an association of all those SNPs [67], but a second study on a Chinese Han population detected an association of only rs9296249 [68], while a third study on a Polish population did not find any significant association [69].

2.2.3.2 Single Positive Findings

Many other single nucleotide polymorphisms have been discovered and proposed as susceptibility factors in the development of Tourette Syndrome. In the following section they are grouped according to the different biological pathways that may or may not be involved in TS pathogenesis.

2.2.3.2.1 Glutamate

Several genes are linked to the glutamatergic pathway, thus supporting the theory of glutamate and its involvement in Tourette Syndrome [70].
For instance, is the case of cadherin 2, type 1, N-cadherin neuronal (*CDH2*). It regulates the growth and navigation of axons, the correct function of synapses [71] as well as the traffic of glutamate [72]. A genetic study performed on a cohort of both OCD and TS patients detected a positive, but not significant, association of four SNPs with TS [73]. Although none of these SNPs were statistically significant, rs201333291 was the most interesting because it was found in 3 TS patients but not among the controls and because further studies showed that rs201333291 resulted in reduced *CDH2* protein expression [73].
Supporting the theory of glutamate is also the case of rs1805476 and rs1805502 located on glutamate receptor, ionotropic, N-methyl D-aspartate 2B (*GRIN2B*) linked to Tourette in a Chinese Han population study [74]. It encodes for the subunit 2 of the NMDA receptor channels and acts as agonist binding site for glutamate. It is expressed in the hippocampus, basal ganglia, and cerebral cortex where it acts as excitatory neurotransmitter receptor [75].
One more case is represented by rs5751876 located on the adenosine A2a receptor (*ADORA2A*) gene and rs2228079 situated on adenosine A1 receptor (*ADORA1*) which have been associated to Tourette in a Polish population study [76]. The heterodimer formed by the A1-A2 receptors regulates the striatal glutamatergic neurotransmission therefore an abnormal regulation caused by SNPs in these two genes might represent a risk factor for TS [77]. The A1 receptor is widely distributed in the brain, where it acts as an inhibitor of synaptic transmission and neuron hyperpolarization, while the A2 receptor

is highly expressed in the striatum, where it functions as facilitator of the transmitter release and as regulator of the sensorimotor integration into basal ganglia [78].

2.2.3.2.2 Oxidative Stress

One interesting theory about Tourette Syndrome is the involvement of oxidative stress. Indeed, few susceptibility genes are involved in the detoxification pathways, and it is possible to speculate and to factor in also oxidative stress in the occurrence of the pathology. However, further studies and more strong findings are needed to fully accept this hypothesis.

The first gene involved in the theory is poly (ADP-ribose) polymerase 1 (*PARP1*) which encodes for a chromatin-associated enzyme that modifies various nuclear proteins by poly(ADP-ribosyl)ation. It has been reported that rs1805404 is susceptibility factor for TS [79].

The second gene around which the oxidative theory is built is X-ray repair complementing defective repair in Chinese hamster cells 1 (*XRCC1*), which is known to interact with PARP1 in the base excision repair pathway after oxidative stress. A study on Chinese Han population linked this gene to TS through rs25487 [80].

The third and last gene is glutathione S-transferase pi 1 (*GSTP1*) which has been associated to TS in a Taiwanese study through rs6591256 [81]. *GSTP1* is involved in the detoxification process by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione [82].

2.2.3.2.3 Dopamine & Serotonin

Compelling evidence (as also from genetic studies) supports the idea that both dopaminergic and serotoninergic pathways play a role in Tourette Syndrome [83] and there are some single positive finding falling under this theory.

Some examples are the glial cell derived neurotrophic factor (*GDNF*), the solute carrier family 6 (neurotransmitter transporter) member 4 (*SLC6A4*) and the tryptophan hydroxylase (*TPH2*) genes.

GDNF has been recently associated to TS through rs3096140 [84]. It promotes the survival and differentiation of dopaminergic neurons and it prevents the apoptosis of motor neurons [85].

SLC6A4 (serotonin transporter) expression is controlled by the SERT-linked polymorphic region (5-HTTLPR), which consists of a 43bp (VNTR) in the promoter region that is characterized by a short (S) and a long (L) allele. It has been shown how rs25531, the long allele, might be associated to Tourette Syndrome [86]. The *TPH2* gene codes for the tryptophan hydroxylase enzyme, which is responsible for the synthesis of serotonin in the brain [87] and a variant of this gene has been associated with TS [88].

2.2.4 Disentangling the Genetic Complexity of Tourette Syndrome

It is important to pause for a moment and look again at the multiple positive genetic findings described above.

It is not possible to not realize how difficult it is to study and to identify a positive association in a complex disorder like TS. It is one of the reasons that lead to the establishment of an extensive research network (TS-EUROTRAIN) able to dig in the genetic background of Tourette Syndrome with sufficient tools and resources to overcome issues like population stratification, sample size and statistical power [6]. It is very unlikely that one single variation might cause all the variety of symptoms that appear in the patients. In my opinion, the fragmentation of these findings resembles the genetic complexity of Tourette Syndrome and supports the theory that a combination of small effects leads to the manifestation of the phenotype. It is also important to notice that in most of the studies mentioned above, the genetic findings have not been yet replicated partly because they have been found in small samples that may suffer from population stratification. Moreover, not many of the SNPs findings have been supported by follow-up experiments to actually check the biological relevance of the variations identified.

Small sample size represents also a major problem in the TS dataset used for the genetic association study of my PhD project. In order to maximize the chances of finding something statistically significant, I decided to focus on the 3'UTR genes of TS candidate genes for the following reasons:

- there are few studies reporting association within the 3'UTR
- the presence of SNPs in the 3'UTR might have an important biological relevance in case it is located within a miRNA binding site
- the possibility to perform follow-up studies

2.3 Epigenetic Background of Tourette

Tourette Syndrome is not only about genetics.
In the last years there has been a growing consensus that epigenetics might also contribute to the development of the pathology. Epigenetics usually refers to those changes, such as DNA methylation, histone modifications and microRNA regulation, in the gene expression or cellular phenotype that do not alter the sequence of the DNA [89]. Since these modifications are both cells and time specific it becomes extremely important to study not only the exact tissue involved in the pathogenesis of the disorder, but also at the correct time. Moreover, these modifications are usually dynamic and influenced by both internal and external stimuli [90].
Tourette Syndrome clearly appears to be a good candidate for epigenetic mechanisms: symptoms appear and disappear based on the emotional status of the patients with a different intensity and frequency, and the time lapse of the disorders is scattered and different for every patient.
It is known that external factors like psychological stress, maternal smoking during pregnancy, the age of the father, birth weight and exposure to medication in utero represent a risk for TS development [91]. The appearance of TS might be a consequence of a pediatric autoimmune neuropsychiatric disorder associated with streptococcal infection (the PANDAS hypothesis) [92]. Some direct links to Tourette Syndrome and DNA methylation or microRNA regulation have been recently reported [19].
Epigenetic changes are cells specific and Tourette Syndrome is a neurodevelopmental disorder. The difficulty to access human brain samples represents a major drawback in the understanding of epigenetic alterations in TS pathophysiology and it makes the availability of animal models a key component in scientific studies. For these reasons, TS-EUROTRAIN Network [6] had 2 PhD projects focused only on the establishment of

new animal models of TS in order to have a direct access to brain tissues, during childhood and adolescence, involved in the pathology (see below).

2.3.1 The role of DNA Methylation in epigenetic regulation

DNA methylation is the predominant epigenetic modification in eukaryotic DNA. It refers to a covalent addition of a methyl (CH_3) group to the 5'C of a cytosine residue located in area of the genome rich in cytosine-phosphate-guanine (CpG) dinucleotides.
In the genome, there are stretches of DNA with a very high density of CpG dinucleotides (CpG islands) usually found in an unmethylated status [93]. In those area, it is possible to find approximately 70% of promoters, key regulatory regions in the modulation of genes transcription [94]. The CpG islands associated with promoters are usually conserved across species because of their role in transcriptional processes [95]. In particular, they regulate both the chromatin structure as well as the binding of transcription factors. Indeed, the addition of a methyl group to the DNA leads to the formation of a very tight structure (heterochromatin) usually wrapped around histones that is difficult to access for transcriptional factors and other regulatory units.
DNA methylation represses transcription directly, by inhibiting the binding of specific transcription factors, and indirectly, by recruiting methyl-CpG-binding protein [96]. Moreover, at least in mammals, methylation of DNA is associated with other processes like genomic imprinting, X-chromosome inactivation, repression of transposable elements, aging, and carcinogenesis [97].
DNA methylation is catalyzed by a family of enzymes called DNA methyltransferases (DNMTs) which either create or preserve the methylation profile. In mammals, this family has three major members: DNMT1, DNMT3A and DNMT3B. These proteins have a very similar structure containing a catalytic C-terminal domain and a regulatory N-terminal domain.
DNMT1 is a maintenance methyl-transferase that binds to hemi methylated DNA strand in order to preserve and restore the pattern of methylation after DNA replication processes [98]. Indeed, DNMT1 is located at the replication forks, where it binds the newly synthesized DNA and it catalyzes the reaction to restore the original methylation pattern [99]. In contrast, DNMT3A and DNMT3B are de novo methyltransferases capable of

originating a new pattern of methylation. DNMT3L is catalytically inactive but stimulates de novo methylation by binding to DNMT3A/B and it is also thought to be required for the establishment of maternal genomic imprints [100].

On the other hand, DNA demethylation is catalyzed mainly by members of the ten-eleven-transferase (TET) enzyme family through the hydroxylation of the methyl group. The reaction leads to the formation of 5-hydroxymethyl-cytosine (5-hmC) [101, 102].

The methylation of DNA usually involves a few percentage (between 3 and 7%) of the genomic cytosines, while the 5-hMC involves only 0.01–1% of all cytosines [103].

During development, differentiating cells undergo dynamic methylation changes at genome wide level before reaching the adult cell type. Similarly, the induction and differentiation of stem cells is followed by rapid changes in DNA methylation [19]. Moreover, DNA methylation results also in response to external factors like hormonal, metabolic or early childhood stress. It eventually leads to the silencing of gene expression. Same fallout has been observed in presence of toxic molecules or viral infections [102].

Epigenetic modifications do play a pivotal role in brain plasticity as well as different central nervous system processes: the differentiation of neuronal stem cells [104], the molecular, hormonal and behavioral response to environmental stimuli [105-107] and the plasticity of synapses [108]. It is therefore important to establish and also to maintain the cell-specific methylation pattern during the early stage of brain development [109].

To further support the importance of DNA methylation in brain development there are the findings of a dynamic expression of *DNMT3A* and *DNMT3B* [110] as well as the appearance of neuronal deficits in case of mutation in the methyl-CpG-binding protein 2 (*MECP2*) [111, 112]. Indeed, mutations in *MECP2* have been associated to autism spectrum disorder and Rett Syndrome [113-115].

Moreover, different studies found evidences of abnormal methylation levels linked to the manifestation of neurological symptoms in Schizophrenia [116, 117], Parkinson's disease [118, 119] and, more recently, Tourette Syndrome [120].

However, most of the studies published so far are performed on small sample size or on surrogate tissues because it is not possible to access the brain tissue at the time of the manifestation of tics. In conclusion, there is a major need for more studies to untangle the involvement of epigenetics in the development of neurological disorders.

2.3.1.1 DNA Methylation and Tourette Syndrome

To date, very few papers have studied methylation changes in relation to TS. There is a study that provides a direct link between DNA methylation of *DRD2* and TS [120]. Indeed, the study was able to show a positive correlation between high methylation level of the *DRD2* gene and the severity of tics. However, the study involved a group of adult patients and detected also increased methylation along with age and antipsychotic medication [120]. The authors are speculating that hyper-methylation of *DRD2* might lead to dysfunction in the dopaminergic system, thus leading to an enhanced locomotor activity throughout the indirect CSTC pathway.

The second study is the first Epigenome Wide Association Study (EWAS) investigating DNA methylation differences between controls and patients from the Netherlands Twin Registry with tic phenotype [121]. It showed an enrichment of differentially methylated neural genes previously linked to neuropsychiatric disorders or with brain specific functions (see below).

2.3.2 The role of microRNAs in the regulation of gene expression

The microRNAs (miRNA) are a small, non-coding RNA molecule found in plants, animals and some viruses, which function in transcriptional and post-transcriptional regulation of gene expression.

Most of the miRNAs are highly expressed in the human brain, where they exert their function by modulating cell survival, neurite projection, and synaptic plasticity. It is not surprising, given their expression and function, that miRNAs have an important role in many psychiatric disorders [122].

The biogenesis of microRNAs (Fig. 2) is a process that starts in the nucleus and ends in the cytoplasm with a cleavage point in each nuclear localization. The final product is a single strand with a length of ~22 nucleotide that subsequently affects the expression of genes. In the first step, the RNA Polimerase II transcribes the coding gene of a miRNA into a prototype (pri-miRNA) with a very distinctive hairpin structure and a terminal loop [123]. Subsequently, during the first cleavage step, the hairpin structure is cut originating the pre-miRNA, a prototype with a length of ~70 nucleotide which is then exported into

the cytoplasm [124]. In the cytoplasm, the pre-miRNA is cleaved for the second time to originate a precursor with a length of ~22 nucleotide, generally called miRNA-miRNA duplex. One strand of this duplex is placed into a large multi-protein miRNA ribonucleoprotein complex (RISC complex), while the remaining strand is degraded. The strand incorporated into RISC, guides the subunit to a messenger RNA (mRNA). Based on a base-pair interaction, a 6–8 nucleotide domain located at the 5′ end of the miRNA, the miRNA might bind the 3'UTR of a mRNA leading to a reduction of the gene expression through the repression of translation or the degradation of mRNA [125].
Therefore, the presence of SNPs in the 3′UTR could be important because it might either destroy or create a binding site for miRNAs:

a. it can partially or completely disrupt the miRNA binding site, thus resulting in higher expression of the target gene
b. it could enhance the binding of a miRNA to the 3′UTR region
c. it may create a novel binding site for miRNAs

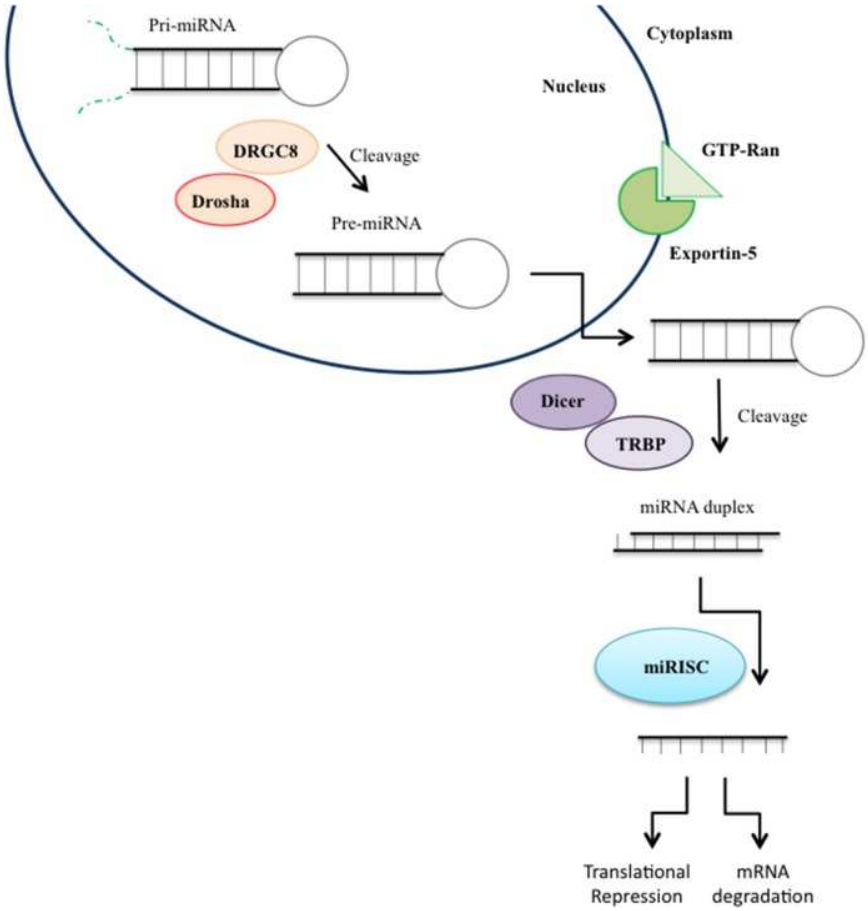

Fig. 2 from [19]

The biogenesis of miRNAs: MicroRNA (miRNA) genes are transcribed as primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II) in the nucleus. The pri-miRNAs are subsequently cleaved to originate pre-miRNAs, which are then exported to the cytoplasm and cleaved to produce a miRNA duplex. One strand of the mature miRNA is loaded into the miRNA-induced silencing complex (RISC) and it leads to mRNA degradation or translational repression.

2.3.2.1 MicroRNAs and Tourette Syndrome

The finding of Abelson et al. 2005 [51] is the only genetic study with a direct link between miRNA and Tourette Syndrome. In this paper, the author reported an interaction between mir-189 and *SLITRK1* in TS patients carrying one noncoding sequence variant, var321, that changes one nucleotide in the sequence of the miRNA binding site (G:U into A:U). This variation results in a very highly conserved binding site for microRNA and it allows mir-189 to strongly repress *SLITRK1* protein expression [51].
It had been 10 years before the paper of Rizzo et al. 2015. The study is focused on miRNA expression profile in serum of six TS patients and three controls and it showed a significant under-expression of mir-429 TS patients [126].

2.3.3 Increased Effort to Study Methylation and Tourette Syndrome

It appears evident that epigenetic studies and Tourette Syndrome have a very long way to go and, as previously said, TS might resemble some epigenetic peculiarities. Indeed, the symptoms wax and wane based on the stress level of the patients and so do DNA modifications, which can occasionally be very dynamic and change based on environmental stress.
It will never be a possible to directly access the human brain of those young patients. Therefore, scientific studies will always have to rely on animal models since the epigenetic changes are tissue and time specific. It is a field where there is still a lot of knowledge to gain.

2.4 Animal Models

The use of an appropriate animal model to study Tourette Syndrome still represents a big challenge. Indeed, it is very difficult to reproduce in a single animal model all the different clinical manifestations observed in TS patients (for detailed review of TS animal models see [127]). However, they represent the only option to perform invasive biological studies in a living organism.

Animal models are usually characterized by *face validity* (ability to show similar symptoms to the patients' ones), *construct validity* (model developed according to a rationale matching the pathological hypothesis), and *predictive validity* (the model responds to a treatment similarly to patients).

The existing animal models of TS available today do not possess all the three validity components but are mostly based on a single component:

1) based on genetic findings
2) modelling the tic phenotype
3) resembling the TS sensorimotor gating deficit
4) based on environmental factors

2.4.1 Abnormal Involuntary Movements (AIMs)

The availability of an animal model is pivotal in the study of many neurological disorders where it is not possible to access brain tissue involved in the pathology. The model used in this PhD project is called abnormal involuntary movements (AIMs) and it was established by our collaborators at Boehringer Ingelheim pharmaceutical company, in Germany.

This type of model is widely used to study Parkinson's disease (PD) where the chronic administration of L-DOPA often results in the development of AIMs, an important clinical symptom that affects almost 90% of PD patients. However, the pathogenic mechanisms underlying AIMs are not yet understood.

Previous studies showed that chronic L-DOPA administration altered principally the dopaminergic pathway but also several other neurotransmitter systems [128-130]. Indeed,

the administration of L-DOPA activates striatal dopamine D1 receptors (DRD1), resulting in the overstimulation of the "direct" pathway (see above) [131, 132]. The hypersensitivity to dopamine sparks a number of downstream signaling cascades (cyclic AMP (cAMP)-activated protein kinase A (PKA) [133, 134], the phosphatase inhibitor DARPP-32 [134, 135], and the ERK1/ERK2 (ERK1/2) kinases) [136, 137] involved in the manifestation of locomotor activity [138, 139].

2.4.2 Translating AIMs in Tourette Syndrome

Even though the AIMs animal model is very well accepted to study PD, it provides the same pathophysiological condition that leads to the manifestation of tics observed in TS. Indeed, the hypersensitivity to dopamine is a condition observed in both PD and TS patients. Therefore, it has been decided by our collaborators at Boehringer Ingelheim, to translate the AIMs animal model into juvenile rats since Tourette Syndrome is a neurodevelopmental disorder to better study the biological mechanism behind tics as well as to test new drugs (such as Riluzole) able to reduce the manifestation of locomotor activity.

In order to mimic TS pathological conditions, rats were unilaterally injected in the medial forebrain bundle, an area of the brain enriched with projection of dopaminergic neurons of substantia nigra, with a chemical toxin named 6-hydroxidopamine (6-OHDA). Once injected in the brain, the 6-OHDA is selectively absorbed by those neurons expressing the dopamine transporters, such as dopaminergic and noradrenergic neurons, which carry the toxin inside the dopaminergic neurons. The toxin starts to accumulate in the dopaminergic neurons and it eventually leads to the production of reactive oxygen species and to cell death. The absence of dopaminergic inputs results in dopamine hypersensitivity, the same pathological condition observed in TS patients. The subsequent oral administration of L-DOPA, a precursor of dopamine, triggers the appearance of abnormal involuntary movements (AIMs).

On the other hand, Riluzole has been suggested as a candidate drug to treat AIMs since previous studies on rodents provided evidence for the efficacy of Riluzole in the treatment of AIMs [140-142]. As for human studies, Riluzole showed anti-dyskinetic effects in a small pilot study [143], but it was ineffective in another clinical trial [144]. However, the

molecular mechanisms of Riluzole in protecting from the manifestation of AIMs are still unknown. It has been hypothesized that Riluzole might exert its function by acting as a blocker of glutamate, as inactivator of voltage-dependent sodium channels, as obstructer of intracellular processes.
In this PhD project we decided to use the AIMs animal model to study both RNA and DNA of brain to shed some light on the speculated relevance of DNA methylation changes in the development of locomotor activity and to identify drug targets (see below).

2.5 TS-EUROTRAIN Network

TS-EUROTRAIN Network was established with the idea of training 12 Early Stage Researchers (ESRs) to collaborate on the investigation of Tourette Syndrome and to share the knowledge deriving from imaging studies, animal models, bioinformatics analyses and genetic studies.

2.5.1 TS-EUROTRAIN Tasks

The investigation of environmental and genetic factors in TS:

- identify genes and gene pathways that influence the pathogenesis and clinical course of TS
- explore the involvement of epigenetic phenomena in the onset of symptoms of TS
- explore the complex interaction between the environment, autoimmunity and genetics

The investigation of the neurobiological mechanisms of TS:

- investigate the neurocognitive pathways underlying the broad TS phenotype
- establish patterns of multivariate brain activation and connectivity for TS through neuroimaging studies
- investigate the role of the glutamate metabolism in TS

To translate research findings into clinical applications:

- define endophenotypes and prediction models for TS
- investigate the pathophysiology of the psychotropic compounds that are already in use or under clinical trials for TS
- identify novel targets for drug therapy for TS and related comorbidities

The following PhD work results from an extensive collaboration with several of the PhD students recruited in the TS-EUROTRAIN Network:

1) the animal model was established by our collaborators at Boehringer Ingelheim, Biberach, Germany.

2) the Epigenome Wide Association Study (EWAS) was done with our collaborators at VU Amsterdam University, Amsterdam, The Netherlands.

3) the transcriptome analysis and the molecular landscape was built with our collaborators at Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

I am going to focus on and to describe mainly the parts of the project I was actively working on and gave my substantial contribution. The full description of the methods and results (as well as a proper background) of the projects discussed in this PhD, that are not my expertise, may be found in the respective manuscripts. The only exception involves the methods of the animal model. I was not the one who established it but the model has not been published yet, therefore, I am going to provide more details about it in the methods/results section.
I was also partially involved in the statistical analysis of RNA and DNA data even though I was not the one who did the substantial amount of work since I am not an expert in those bioinformatics analyses.

3. OBJECTIVES

1) Identify new genetic variations that may represent risks for Tourette Syndrome by focusing on the 3'UTR of TS candidate genes.

2) Shed some light on the role of DNA methylation in the development of Tourette Syndrome by performing the first ever Epigenome Wide Association Study (EWAS) on tic disorders benefiting from the TS-EUROTRAIN network resources.

3) Study the transcriptome of striatum of an AIMs animal model to build a molecular landscape aimed to unravel the molecular mechanism of action of Riluzole.

4) Study the methylation pattern of striatum of AIMs animal model at the genome level to identify which changes occur after the administration of L-DOPA or the injection of 6-OHDA.

4. METHODS

4.1 Genetic studies of Tourette Syndrome

4.1.1 In silico work

I originally obtained a list of TS candidate genes by performing a literature research on PubMed using the terms "Tourette Syndrome" or "TS" combined with "gene" or "genetics" and also by using a software called HugeNavigator [145]. Subsequently, I focused the attention on SNPs which are located in the 3'UTR regulatory sequences of TS candidate genes and also predicted (using the PolymiRTS database) to alter the seed sequence of miRNAs binding site. Thirty such SNPs were selected and successfully genotyped using our customized TaqMan® OpenArray® genotyping chips (Table 2).

Table 2: List of SNPs selected for the OpenArray® genotyping chip

Gene	Gene name	SNP	Minor Allele Frequency	SNP found in PolymiRTS
AADAC	Arylacetamide deacetylase	rs1042201	0.49 (A)	
ACP1	Acid phosphatase 1, soluble	rs6855	0.20 (G)	Yes
CNR1	Cannabinoid receptor 1	rs4707436	0.24 (A)	Yes
		rs806368	0.28 (C)	Yes
CNTNAP2	Contactin associated protein-like 2	rs1062072	0.46 (A)	Yes
		rs2530310	0.42 (T)	Yes
		rs2530311	0.47 (A)	Yes
		rs987456	0.30 (C)	Yes
COMT	Catechol-O-methyltransferase	rs165599	0.47 (G)	Yes
		rs165728	0.16 (C)	
DRD2	Dopamine receptor D2	rs6276	0.48 (T)	Yes
		rs6278	0.20 (A)	Yes

GDNF	Glial cell derived neurotrophic factor	rs2973051	0.34 (C)	Yes
		rs3749692	0.46 (A)	Yes
		rs62360370	0.04 (A)	Yes
HTR2A	5-hydroxytryptamine receptor 2A	rs3125	0.14 (G)	Yes
ILR1N	Interleukin 1 receptor antagonist	rs4252041	0.02 (T)	Yes
IMMP2L	Inner mitochondrial membrane peptidase subunit 2	rs1044729	0.34 (C)	
		rs17158195	0.13 (T)	
		rs7795011	0.46 (G)	
LHX6	LIM homeobox 6	rs3750486	0.08 (A)	Yes
		rs74370188	0.01 (A)	Yes
MEIS1	Meis homeobox 1	rs72824830	0.02 (G)	Yes
NTN4	Netrin 4	rs1052651	0.38 (A)	Yes
		rs8699	0.43 (G)	Yes
SLC6A3	Solute carrier family 6 member 3	rs11564774	0.23 (G)	
		rs7732456	0.07 (C)	
SLITRK1	SLIT and NTRK like family member 1	rs3737193	0.03 (G)	Yes
		rs41557622	0.02 (T)	Yes
TNF	Tumor necrosis factor	rs3093665	0.02 (C)	Yes

4.1.2 Samples

DNA samples are part of the TSGeneSEE consortium collection (the Tourette Syndrome Genetics - Southern and Eastern Europe Initiative), consisting of 148 families and 290 case-control samples from Hungary, Italy, Poland, Greece and Albania. The assessment was performed by on-site clinicians using the tools provided by the TS Association International Consortium for Genetics. Diagnosis of TS was ascertained according to DSM-IV-TR criteria for Italy, Hungary, Albania and Greece, and DSM-IV for Poland. The collection of TSGeneSEE samples was coordinated and approved by the Democritus University of Thrace Research Ethics Committee, and a written informed consent was

obtained from all participating individuals or their parents. The average age at onset of the children (154 males, 152 females) involved in the study was 5.7 ± 2.19 years.
Regarding the Transmission Disequilibrium Test (TDT), the sample consisted of "*trios*" where we have records of the complete family (child, mother and father) and the "*incomplete trios*" where one of the parents is missing from the dataset. The combined dataset consisting of both full trios and incomplete trios will be referred as "*families*".

4.1.3 Genotyping

Genotyping of samples was performed using TaqMan® OpenArray® Genotyping System [146]. The processing of raw data was performed by the TaqMan® Genotyping Software (Applied Biosystems®). As a quality control step, 10% of samples were re-genotyped to avoid any batch effects and samples that failed to pass the genotyping rate of 90% were removed from the analysis. The final analysis consisted of 141 families (full and incomplete trios) and 266 samples (141 cases, 125 controls).

4.1.4 Statistical Analysis

Standard case-control association analysis was conducted in PLINK [147] using $\chi 2$ test comparing SNP frequencies within cases to that of controls. To avoid artifacts that might arise due to population structure we performed basic $\chi 2$ association with respect to TS separately for the different populations. The individual population association results were further meta-analyzed using the METAL software in a case-weighted fixed-effect model on p-values [148]. To correct the results for multiple testing 100,000 permutations were applied with the Monte Carlo Permutation (MCPerm method) [149].
Family-based association was performed using the family-based Transmission Disequilibrium Test (TDT) option in PLINK software [147]. Families were checked for Mendelian errors and samples with an error rate above 10% were discarded from further analysis. Empirical significance levels were generated with PLINK using max (T) permutation methods set to 1000 permutations.

4.1.5 Real-Time PCR

On the same dataset that I used for the 3'UTR Open Array, I also performed genotyping of some of the candidate genes by Real-Time PCR. **Table 3** shows the SNPs investigated.

Table 3: List of SNPs analyzed by Real-Time PCR

Gene	Gene name	SNP	Genotype
GDNF	Glial cell derived neurotrophic factor	rs11111	A/G
		rs1549250	G/T
		rs2910702	A/G
		rs2973033	A/G
		rs3812047	A/G
		rs1981844	C/G/T
SLC6A3	Solute carrier family 6 member 3	rs27072	C/T
TNF	Tumor necrosis factor	rs361525	A/G
WFS1	Wolframin ER transmembrane glycoprotein	rs1046322	A/G
		rs1046320	A/G

4.2 Epigenetics studies of Tourette Syndrome

4.2.1 Samples

The subjects in this study were participants in the NTR biobank Project. The analysis consisted of 1678 individuals (twins, siblings, and parents) from 1057 families [121].

4.2.2 Infinium HumanMethylation450 Bead Chip Kit (Illumina 450K)

DNA methylation was assessed with the Infinium HumanMethylation450 Bead Chip Kit Array which covers 17 CpG sites on average per gene distributed across the promoter, 5'UTR, first exon, gene body, and 3'UTR.

4.2.3 Statistical Analysis

EWAS (epigenome-wide association study) was performed using linear regression under an additive model correcting for principal components and covariates and CpG sites with p-value < 1.2*10-7 were considered statistically significant [121].

4.2.4 Enrichment of Gene Ontology terms

The gene ontology enrichment analysis was performed by ranking all the methylation sites based on their p-value and the resulting ranked gene list was supplied to the online software tool Gorilla [150]. False Discovery Rate (FDR) q-value < 0.05 was considered for a GO term to be statistically significant. The literature research to investigate the biological relevance of the findings was performed using PubMED and GeneCards.

4.3 Animal model exhibiting abnormal involuntary movements (AIMs)

The animal model (Fig. 3) was established by our collaborators at Boehringer Ingelheim, Biberach, Germany.

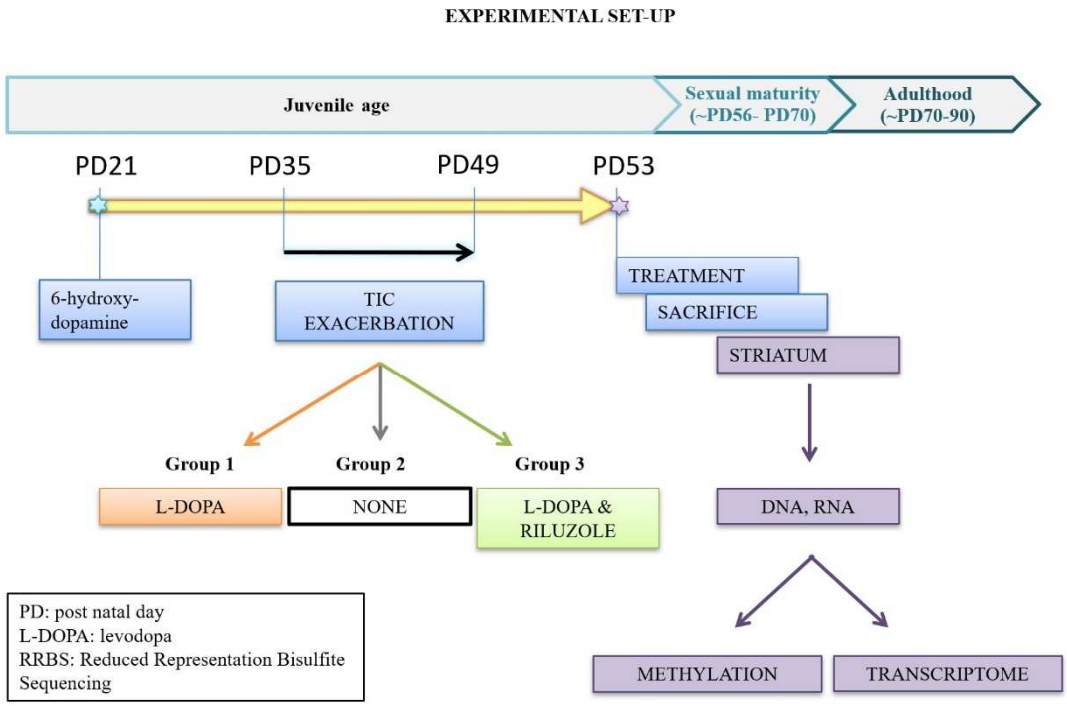

Fig. 3: Schematic diagram of the protocol employed including the time course of the experiment and the different assays that were used.

4.3.1 Stereotaxic surgery

The rats underwent stereotaxic surgery with 6-hydroxydopamine (6-OHDA) at post-natal day (PND) 21. Once injected in the brain, the toxin starts to accumulate in the dopaminergic neurons and it eventually leads to the production of reactive oxygen species and to cell death.

4.3.2 Animal treatment

The treatment administration (three times a week) started two weeks after the surgery, and the animals were randomly located into three groups (n = 12). The first group was chronically administered with a solution of L-DOPA (orally). The second group (control) was administered with only saline. The third group was chronically administered with L-DOPA + Riluzole, a GABAergic/antiglutamatergic drug.

4.3.3 Phenotype scoring

During every treatment administration, rats were individually observed for 1 minute every 20 minutes and abnormal involuntary movements (AIMs) of the limb, mouth, and body axis were scored on a scale from zero to four (0 = absent; 1 = occasional; 2 = frequent; 3 = continuous but interrupted by sensory distraction; 4 = continuous, severe, not interrupted by sensory distraction) [151].

4.3.4 Samples extraction

At post-natal day 53 (PND 53), the rats were administered one final treatment and sacrificed 2 hours later by a guillotine. The brain was removed, lesioned and non-lesioned striata were extracted, frozen in liquid nitrogen, and stored at – 80°C.

4.3.5 RNA sequencing and data analysis

Total RNA was extracted from striatum tissue from eight animals of each group using the QiagenAllPrep Kit (Qiagen), according to the manufacturer's instructions. RNA was quantified using the NanoDrop 1000 Spectrophotometer and Bioanalyzer 2100 (Agilent). Samples with RIN value above 8.0 were used for transcriptome analysis. Library preparation for sequencing was done using 200 ng of total RNA input with the TrueSeq RNA Sample Prep Kit v3-Set B (RS-122-2002, Illumina Inc, San Diego, CA) and the sequencing was then performed as 78 bp, single reads and 7 bases index read on an Illumina HiSeq3000 instrument using the TruSeq SBS Kit HS- v3 (50-cycle) (FC-401-

3002, Illumina Inc, San Diego, CA). Statistical analyses were performed using R (www.r-project.org) and the Bioconductor package limma-voom [152]. The Benjamini-Hochberg method was used to correct for multiple testing, and only protein-coding genes with adjusted P-value < 0.01 were used for further analyses.

The upstream regulator and gene enrichment analyses were done using Ingenuity Pathway Analysis (IPA) (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/). For each upstream regulator, IPA calculates a p-value of overlap (measuring the statistical significance of the overlap between the dataset genes and all genes that are regulated by the upstream regulator) and a z-score (reflecting the inhibition or activation of the upstream regulator-dependent effects on target gene expression).

4.3.6 qPCR validation of selected CREB1 targets

To check the reliability of the RNA sequencing data, we performed qPCR on 10 randomly chosen CREB1 target genes (Table 4). 100ng of RNA were reverse-transcribed to cDNA using the RT^2 First Strand Kit (Qiagen, Cat. number 330404) according to the manufacturer's instructions. Three-step qPCR (95°C for 10min, followed by 40 two-step cycles of 95°C for 15s, 60°C for 30s, and the generation of melting curves from 70 °C to 95 °C; Rotor-Gene Q 1000, Qiagen) was performed using RT² SYBR Green ROX™ qPCR Mastermix (Qiagen, Cat. number 330521) and RT^2 qPCR primer Assays provided by Qiagen. The housekeeping genes *Bcap29* and *Cdkn1b* were used as reference for normalization of gene expression, and a Student's t-test was used to assess statistical significance.

Table 4: List of the gene and the primers (RT² qPCR Primer Assay by Qiagen) used for the qPCR validation experiment of mRNA data

Gene Symbol	Catalog number	RefSeq Accession
JUN	PPR53221A	NM_021835.3
NPAS4	PPR52619A	NM_153626.1
GPR3	PPR52464A	NM_153727.1
NR4A3	PPR51343E	NM_017352.1

SRXN1	PPR50360B	NM_00147858.3
GADD45G	PPR46380A	NM_001077640.1
PVR	PPR44991A	NM_017076.2
ATF3	PPR44403B	NM_012912.2
REM2	PPR06549B	NM_022685
EHD4	PPR43831A	NM_139324
Housekeeping genes		
Gene Symbol	Forward Primer	Reverse Primer
BCAP29	AGAAGGCTTCCGATGCCCT	TGCTCTTTCAGGAGTCGGTCA
CDKN1B	CAGACGTAAACAGCTCCGAATT	CTCAGTGCTTATACAGGATGTC

Table 4 from [151]

4.3.7 Reduced Representation Bisulfite Sequencing (RRBS)

Reduced representation bisulfite sequencing (RRBS) is a high-throughput technique used to analyze genome-wide DNA methylation profiles at the single nucleotide level of 4 million CpGs (https://www.diagenode.com/en/p/premium-rrbs-kit-x96-96-rxns). In human, mouse and rat research, the protocol provided by Diagenode allows to replicate and compare DNA methylation levels in different experimental groups at an affordable price. Given the several experimental groups included in the methylation studies (some of which are not included in this PhD dissertation) we decided to use this particular protocol.

4.3.8 RRBS protocol

Striata from rodent animal models were processed using Premium RRBS kit x24 (Diagenode). DNA was extracted using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). DNA fragmentation was checked on 1% agarose gel and only high-quality

DNA was used for preparation of Reduced Representation Bisulfite Sequencing libraries using Premium RRBS kit (Diagenode).

Briefly (Fig. 3), 100ng of DNA was digested with MspI restriction enzyme which cuts at C^CGG sites regardless of the methylation status. Subsequently, the samples were treated with an enzyme that adds flanking ends and during this reaction, some spike in control to check the bisulfite conversion efficiency was also added. In the next step, a specific string of adaptors (6 base pair) was added to each sample followed by a size selection of the fragments (200-1200 bp). Sample concentration was then checked by quantitative PCR and up to 6 samples were pooled together using RRBS pooling algorithm provided by Diagenode. The software compares the Ct values and it calculates their relative concentrations 2^(-dCt). Bisulfite conversion was performed on pooled samples, in this step unmethylated cytosine is converted into uracil while methylated cytosine remained cytosine. Libraries were then ready for a quantification step followed by an enrichment PCR. Finally, the enriched PCR products were recovered, and the concentration was measured by Qubit dsDNA HS Assay (Life Technologies). The library profile was checked on Bioanalyzer 2100 (Agilent) before being sequenced on Illumina HiSeq2000.

4.3.9 RRBS data analysis

Illumina reads were quality checked with the FastQC (v0.10.0) software. The reads were trimmed by trimgalore (v0.4.0) and mapped to rn6 genome using Bismark (v0.14.3.) tolerating one non-bisulfite mismatch per read (-n 1 option). After mapping, the sorted sam files were used by methylKit (v1.0.0) for further analysis. First, methylated and unmethylated Cs in CpG context were checked and Cs with <=10 reads were used for further analyses. Similarity of samples were checked by pairwise Pearson's correlations. Differentially methylated sites (DMSs) were considered significant only after both overdispersion correction and Chi square test with q <0.01. DMSs were annotated according to the type of genomic region (promoter, exon, intron, intergenic or CpG islands, shores, others). Hypo and hypermethylated genomic positions were further annotated to obtain the nearest ENTREZ and RefseqIDs, Gene Names, etc.).

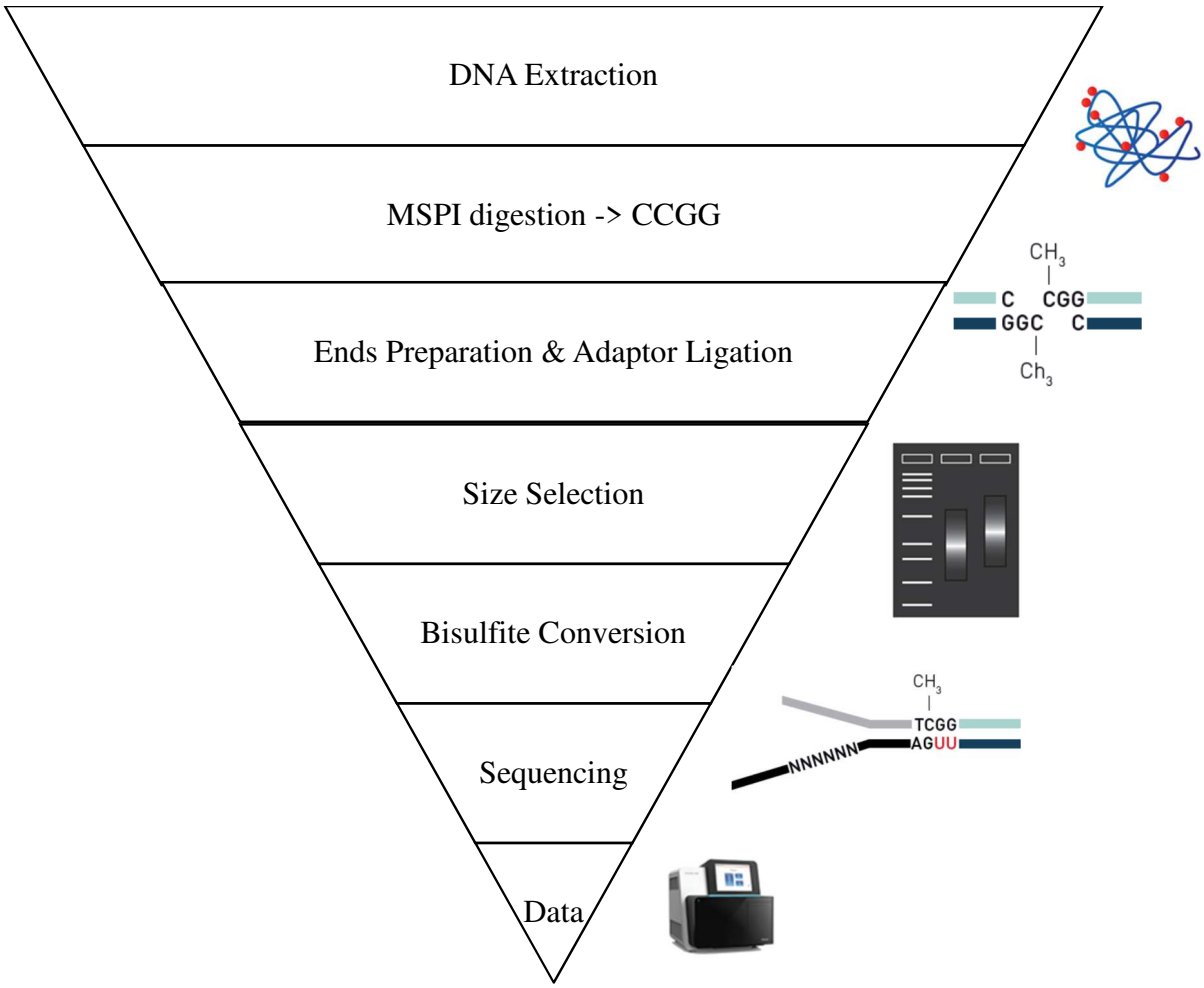

Fig. 4: Reduced Representation Bisulfite Sequencing (RRBS) protocol workflow. The first step of the protocol is DNA extraction followed by a step of enzyme digestion and adaptor ligation. The samples are then selected based on the size (200-1200 bp). Samples undergo bisulfite conversion, sequencing and data analysis to identify genome wide methylation changes.

5. RESULTS

5.1 Genetic studies of Tourette Syndrome

5.1.1 Genetic variation identified by meta-analysis

Table 5 shows the top results ($p < 0.1$) from the meta-analysis which lead to the identification of two significant SNPs that might represent risk factors for TS. The strongest signal was rs3750486 ($p = 0.021$) in the *LHX6* gene, while the other significant SNP was rs7795011 ($p = 0.029$) in *IMMP2L*. Notably, the findings did withstand multiple correction tests.

Table 5: List of the top hits (p-value < 0.1) of the meta-analysis

Gene	SNP	1st Allele	2nd Allele	Frequency of affected allele	P
LHX6	rs3750486	A	G	0.1075	**0.021***
IMMP2L	rs7795011	T	G	0.5483	**0.029****
AADAC	rs1042201	A	G	0.4648	0.077
GDNF	rs2937051	T	C	0.6952	0.095

*permutation p = 0.00136 **permutation p = 0.00935

5.1.2 Over-transmission of alleles in TS patients

Similar to the meta-analysis, we performed TDT on parent-child trios within each individual population as well as for the complete TSGeneSEE dataset (141 families). Table 6 shows the results of the full trio TDT test for each individual population.

Table 6: The results of the TDT test with a focus on the top hits of the meta-analysis.

		Poland (23 trios)				Italy (40 trios)				Hungary (19 trios)				Greece (9 trios)			
GENE	SNP	Ov.T	T:U	$\chi 2$	P	Ov.T	T:U	$\chi 2$	P	Ov.T	T:U	$\chi 2$	P	Ov.T	T:U	$\chi 2$	P
AADAC	rs1042201	G	9:8	0.059	0.808	G	27:13	4.9	**0.027**		5:5	0	1	A	3:0	3	0.083
GDNF	rs2973051	C	10:8	0.222	0.637	C	18:16	0.118	0.732	T	8:7	0.067	0.796	C	6:3	1	0.317
IMMP2L	rs7795011	G	11:7	0.889	0.346	G	25:16	1.976	0.156	G	7:6	0.077	0.782	T	8:0	8	**0.005**
LHX6	rs3750486	A	5:0	5	**0.025**	G	15:2	9.941	**0.002**	A	1:0	1	0.317	G	1:0	1	0.317

Ov.T = over-transmitted allele, T:U = transmitted:untransmitted, $\chi 2$ = chi-square value,

P = unadjusted p-value

When looking at individual populations, rs3750486 in *LHX6* showed a slight but not significant over-transmission of the A allele in the Polish population (p = 0.025, $\chi2$ = 5.0) and a significant transmission of the G allele in the Italian population (p = 0.002, $\chi2$ = 9.941) which remained significant also after 1000 permutation tests (p = 0.037). For rs7795011 in *IMMP2L*, we observed a nominally significant over-transmission of the T allele in the Greek population (p = 0.005, $\chi2$ = 8.0) which withstands 1000 permutations test (p = 0.028). For rs1042201 at *AADAC* we also found nominally significant over-transmission in the Italian population (p = 0.027, $\chi2$ = 4.9).

However, the indication obtained from individual population changes dramatically when we analyzed the full TSGeneSEE dataset (all 141 families) which consists of full trios and incomplete trios (Table 7).

Table 7: Results of the TDT test performed on the full TSGeneSEE dataset (141 families) with a focus on the top hits of the meta-analysis

Gene	SNP	Ov.T	T:U	$\chi2$	P	Perm
AADAC	rs1042201	A	31:16	4.787	**0.029**	0.524
GDNF	rs2973051	C	41:37	0.205	0.650	1
IMMP2L	rs7795011	G	44:39	0.301	0.583	1
LHX6	rs3750486	G	7:3	1.6	0.206	0.999

Ov.T = over-transmitted allele, T:U = transmitted:untransmitted, $\chi2$ = chi-square value, P = unadjusted p-value, Perm = 1000 permutations p-value

Indeed, the results of the TDT test on the full dataset show that only allele A of rs1042201 at *AADAC* is over-transmitted in TS patients (p = 0.029, $\chi2$ = 4.787) even though the signal does not survive correction for multiple testing.

It is important to note that *AADAC* gene is the top signal of both the meta-analysis and the TDT test, making this gene a strong candidate for further follow-up studies in TS.

5.1.3 Results of the other genetic variations analyzed

Table 8 shows the results of the genotyping performed by real-time PCR on the TSGeneSEE dataset while Table 9 shows the results of the TDT test.

Table 8: Results of the genotyping experiments done by Real-Time PCR

Gene	SNP	P-value
GDNF	rs1549250	0.320
GDNF	rs2910702	0.385
WFS1	rs1046320	0.386
TNF	rs361525	0.415
WFS1	rs1046322	0.424
SLC6A3	rs27072	0.601
GDNF	rs11111	0.692
GDNF	rs1981844	0.937
GDNF	rs3812047	0.946
GDNF	rs2973033	0.962

As shown in Table 8, we did not find any significant results in the case-control association study. The same result was observed in the TDT test (Table 9).

Table 9: Results of the TDT test of the genes previously analyzed by Real-Time PCR

Gene	SNP	Ov.T	T:U	$\chi 2$	P-value
SLC6A3	rs27072	T	37:26	1.921	0.166
GDNF	rs1549250	C	48:41	0.551	0.458
WFS1	rs1046320	A	49:42	0.539	0.463
GDNF	rs3812047	C	20:16	0.444	0.505
GDNF	rs2910702	C	34:29	0.397	0.528
WFS1	rs1046322	G	18:15	0.273	0.601
TNF	rs361525	G	12:10	0.182	0.670
GDNF	rs11111	C	37:34	0.127	0.722
GDNF	rs2973033	T	30:28	0.069	0.793
GDNF	rs1981844	G	25:24	0.020	0.886

Ov.T=over-transmitted allele, T:U=transmitted:untransmitted, $\chi 2$=chi-square value

The real-time PCR emphasizes one more time how difficult it is to find any significant or positive findings when operating with small and mixed samples. One of the aims of TS-Eurotrain Network is to solve this problem by combining datasets from European laboratories to increase statistical power and to achieve a better representation of population.

5.2 Epigenetic studies of Tourette Syndrome

5.2.1 DNA methylation profile in the blood of TS patients: an EWAS study

Epigenome-wide association study (EWAS) allows the identification of differentially methylated patterns likely associated to a phenotype of interest. We used this approach to investigate a possible correlation between methylation levels and the manifestation of tics. Figure 5 shows the overall distribution of genome-wide methylation level for the individuals (188 cases and 1490 controls) included in the study.

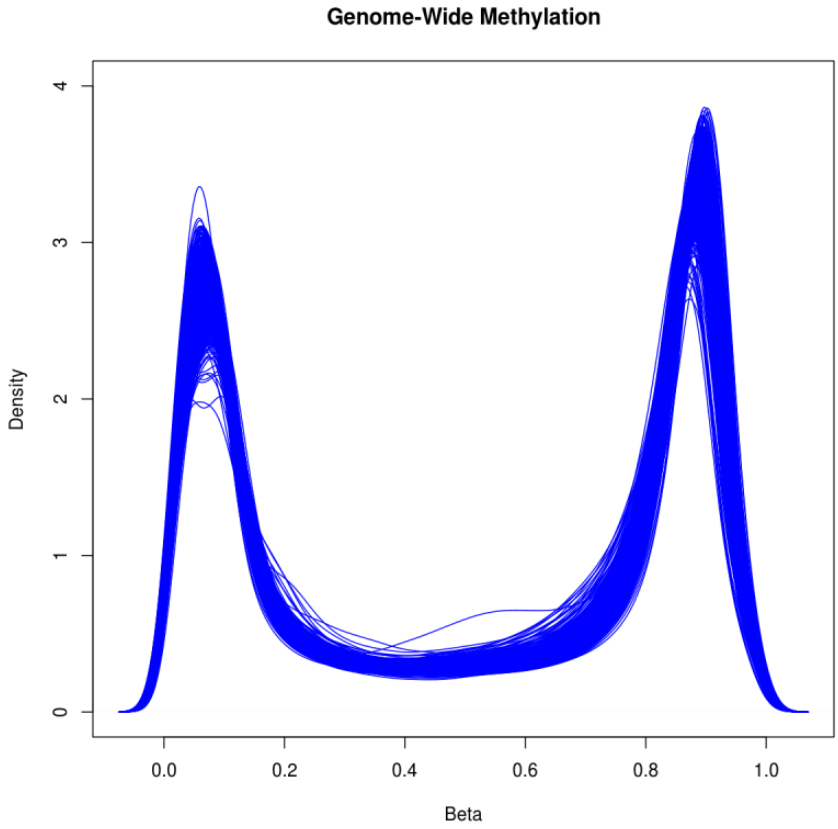

Fig. 5 from [121]

The figure represents the methylation status (min = 0, max = 1) of the CpGs analyzed in the study.

The methylation status of a CpG is usually recorded as β-value that stretches from zero to one respectively for completely un-methylated and methylated status. By looking at this methylation pattern it is important to remember that it represents a single frame taken

at a specific time and it is quite normal to detect CpGs in either fully methylated or non-methylated status.

The EWAS did not detect any significant CpG site (Fig. 6) associated with the tic phenotype. However, it was able to identify 57 CpG sites (Table 10) with a p-value < 0.0001. The permutation test ($p < 0.05$) shows that these CpG sites are not random and might have some relevance for the development of the pathology.

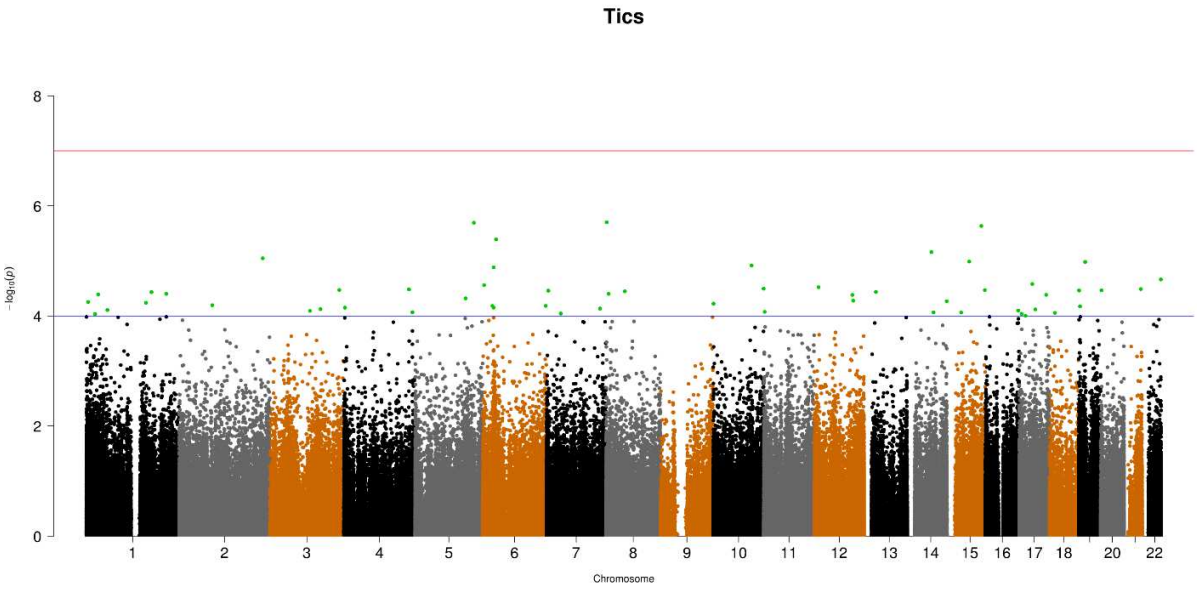

Fig. 6 from [121]

The figure depicts the Manhattan plot of the CpGs analyzed in the study. The red line represents the genome-wide threshold. The top CpG sites are highlighted in green.

It is not surprising to not find any significant CpGs because we need to remember that the samples have a mixed phenotype (severity and duration) therefore to reach statistical significance the analysis would need more samples, both cases and controls, with a more selective phenotype. To achieve a good sample size is an issue that is also hampering the genetic studies since it is very difficult to collect a proper dataset of samples and there are no significant genome-wide findings identified in TS to date

Table 10: The table lists the top CpG sites detected in the EWAS with p-value < .0001.

CpG Site	Chromosome	CpG Location (bp)	P	Gene (Nearby)	Gene Location
cg15583738	8	2176944	1.98E-06		
cg06026425	5	157284650	2.03E-06	*CLINT1*	5q33.3
cg01321816	15	91358514	2.32E-06	*BLM*	15q26.1
cg03573179	6	36165382	4.07E-06	*BRPF3*	6p21.31
cg20519670	14	65172006	6.91E-06	*PLEKHG3*	14q23.3
cg16463697	2	223886480	8.97E-06	*KCNE4*	2q36.1
cg00785856	15	59041883	1.03E-05	*ADAM10*	15q21.3
cg22033061	19	17531746	1.04E-05	*FAM125A/MVB12A*	19p13.11
cg19830950	10	102729375	1.21E-05	*SEMA4G*	10q24.31
cg08093277	6	29595299	1.31E-05	*GABBR1*	6p22.1
cg12961733	22	50165244	2.17E-05	*BRD1*	22q13.33
cg22430950	17	35166190	2.62E-05		
cg16208491	6	4021748	2.76E-05	*PRPF4B*	6p25.2
cg14830166	12	11821908	3.01E-05	*ETV6*	12p13.2

cg23261919	10	135072960	3.20E-05	*ADAM8*	10q26.3
cg08451325	21	44720984	3.25E-05	*SNF1LK/SIK1*	21q22.3
cg15234400	4	174453166	3.29E-05	*NBLA00301/HAND2-AS1*	4q34.1
cg21923992	3	185825749	3.38E-05	*ETV5*	3q27.2
cg07394446	15	100881458	3.39E-05	*ADAMTS17*	15q26.3
cg19391247	20	2360385	3.42E-05	*TGM6*	20p13
cg07975472	19	1503610	3.44E-05	*ADAMTSL5*	19p13.3
cg23572228	7	4923575	3.48E-05	*RADIL*	7p22.1
cg25086136	8	50823124	3.56E-05	*SNTG1*	8q11.21
cg06872019	13	31588778	3.66E-05	*C13orf26/TEX26*	13q12.3
cg20240091	1	175044916	3.69E-05	*TNN*	1q25.1
cg17750334	1	214776613	3.96E-05	*CENPF*	1q41
cg14752139	8	7328654	3.96E-05	*DEFB104B*	8p23.1
cg16650530	1	32538413	4.06E-05	*TMEM39B*	1p35.2
cg23221732	17	72383708	4.14E-05	*GPR142*	17q25.1
cg03704355	12	102270112	4.15E-05	*DRAM/DRAM1*	12q23.2

cg26548492	5	135170171	4.79E-05	*LOC153328/ SLC25A48*	
cg21651356	12	104685539	5.27E-05	*TXNRD1*	12q23.3
cg11597832	14	105993747	5.39E-05	*TMEM121*	14q32.33
cg19403269	1	5569798	5.57E-05		
cg24688563	1	160388964	5.77E-05	*VANGL2*	1q23.2
cg18556420	10	864596	5.99E-05	*LARP5/LARP4B*	10p15.3
cg26116669	2	88654640	6.39E-05		
cg01927730	6	168955764	6.56E-05	*SMOC2*	6q27
cg23066982	6	26204463	6.58E-05	*HIST1H4E*	6p22.2
cg01490283	19	4066033	6.70E-05	*ZBTB7A*	19p13.3
cg21879791	6	29594830	7.07E-05	*GABBR1*	6p22.1
cg15347627	4	2941570	7.08E-05	*NOL14/NOP14*	4p16.3
cg01703966	7	143207845	7.36E-05	*LOC285965*	
cg19511664	3	135685098	7.53E-05	*PPP2R3A*	3q22.2
cg11155621	17	43238118	7.60E-05	*HEXIM2*	17q21.31
cg07477602	1	56961319	7.82E-05	*PPAP2B*	1p32.2

cg00473985	16	87670568	7.94E-05	*JPH3*	16q24.2
cg04747445	3	107241417	8.09E-05	*BBX*	3q13.12
cg27135510	11	2423571	8.43E-05	*TSSC4*	11p15.5
cg02525719	4	183728549	8.59E-05	*ODZ3/TENM3*	4q34.3
cg19497750	14	70588881	8.63E-05	*SLC8A3*	14q24.2
cg22112443	15	37393989	8.66E-05	*MEIS2*	15q14
cg21281009	18	14748298	8.79E-05	*ANKRD30B*	18p11.21
cg01119319	7	38356808	9.04E-05	*TARP*	7p15-p14
cg24419101	17	6484720	9.17E-05	*KIAA0753/TXNDC17*	17p13.1
cg25203007	1	24126017	9.22E-05	*GALE*	1p36.11
cg08490349	17	17086207	9.92E-05	*M-RIP/MPRIP*	17p11.2

Table 10 from [121]

5.2.2 Enrichment analysis of the annotated CpGs

The gene ontology enrichment analysis done using the 57 CpGs listed in Table 10 revealed a great number of GO terms significantly enriched [153]. The *GO Processes* analysis pointed towards developmental process (GO:0032502, FDR q-value = 2.69E-12), cellular developmental process (GO:0048869, FDR q-value = 1.96E-12), single-organism developmental process (GO:0044767, FDR q-value = 2.22E-12). It also picked up, not surprisingly, different brain related processes such as regulation of neuron

projection guidance (GO:0097485, FDR q-value = 5.11E-08), axon guidance (GO:0007411, FDR q-value = 4.89E-08), neuron differentiation (GO:0030182, FDR q-value = 6.24E-08). The *GO component* analysis points towards cell junction (GO:0030054, FDR q-value = 1.09E-05), neuronal structure (GO:0097458, FDR q-value = 1,78E-04), synapse part (GO:0044456, FDR q-value = 1.39E-03), postsynaptic density (GO:0014069, FDR q-value = 3.87E-03) and dendrite (GO:0030425, FDR q-value = 1.05E-02).

The *GO component* analysis gave also terms related to epigenetic. Indeed, it indicated the histone modification, *MOZ/MORF* histone acetyltransferase complex (GO:0070776, FDR q-value=6.20E-03) and *H3* histone acetyltransferase complex (GO:0070775, FDR q-value=5.68E-03) as significantly enriched terms.

5.3 Abnormal Involuntary Movements (AIMs) animal model

The availability of an animal model plays a pivotal role in the study of many neurological disorders by granting access to the brain tissues involved in the pathology. In this PhD project, we used an animal model of abnormal involuntary movements (AIMs) to study both transcriptome and methylome data.

One of the most difficult challenges in interpreting data from this animal model is to distinguish the effect of 6-OHDA injection, the effect of L-DOPA administration, the effect of Riluzole treatment, and the changes that might occur simply as biological noise. In order to solve these questions, we decided to investigate 3 experimental groups (Table 11):

1. **Group 1** - Rats belonging to this group were unilaterally injected with 6-OHDA in the left hemisphere followed by L-DOPA administration, which triggers the tic-like phenotype. This group represents the main phenotype of interest to investigate the effect of L-DOPA administration on hypersensitive striatum.

   Indeed, the 6-OHDA starts to accumulate in the dopaminergic neurons and it eventually leads to the production of reactive oxygen species and to cell death. The absence of dopaminergic inputs results in dopamine hypersensitivity. The subsequent oral administration of L-DOPA, a precursor of dopamine, triggers the appearance of abnormal involuntary movements (AIMs).
2. **Group 2 -** Rats belonging to this group were unilaterally injected with 6-OHDA in the left hemisphere and did not receive any type of additional treatment. This group is to be used for identifying those changes that are due to the lesion but not to levodopa administration.
3. **Group 3 -** Rats belonging to this group were unilaterally injected with 6-OHDA in the left hemisphere followed by L-DOPA administration as well as administration of Riluzole, a drug used on TS patients. The behavioral tests showed a reduction of tic-like symptoms. This group has been used to investigate changes that might occur as a consequence of drug administration.

Table 11: Experimental Groups

Group 1	6-OHDA injection	L-DOPA treatment	X
Group 2	6-OHDA injection	X	X
Group 3	6-OHDA injection	L-DOPA treatment	Riluzole administration

It is possible to make several types of comparisons to detect what changes are due to the different type of substance injected or administered to the animals.
For example:

It is possible to start by looking at each group (1-2-3) and comparing the lesioned side (L) vs. the contralateral side (C):

(1) 6-OHDA vs. no 6-OHDA (Lesion 2 vs Control 2)
--> **effect of the lesion in untreated animals**
(2) 6-OHDA + L-DOPA vs. L-DOPA but no 6-OHDA (Lesion 1 vs Control 1)
--> **effect of the lesion in L-DOPA-treated animals**
(3) 6-OHDA + L-DOPA + Riluzole vs. L-DOPA + Riluzole but no 6-OHDA (Lesion 3 vs Control 3)
--> **effect of the lesion in L-DOPA + Riluzole-treated animals**

It is possible to compare the contralateral side of different groups:

(4) L-DOPA but no 6-OHDA vs. no L-DOPA and no 6-OHDA (Control 1 vs Control 2)
--> **effect of L-DOPA in not-lesioned animals**
(5) L-DOPA + Riluzole but no 6-OHDA vs. no L-DOPA, no Riluzole, no 6-OHDA (Control 3 vs Control 2)
--> **effect of L-DOPA + Riluzole in not-lesioned animals**
(6) L-DOPA + Riluzole but no 6-OHDA vs. L-DOPA but no Riluzole, no 6-OHDA (Control 3 vs Control 1)

--> **effect of Riluzole in L-DOPA-treated, not-lesioned animals**

It is possible to compare the lesioned side of different groups:

(7) 6-OHDA + L-DOPA vs. 6-OHDA (Lesion 1 vs Lesion 2)
--> **effect of L-DOPA in lesioned animals**
(8) 6-OHDA + L-DOPA + Riluzole vs. 6-OHDA (Lesion 3 vs Lesion 2)
--> **effect of L-DOPA + Riluzole in lesioned animals**
(9) 6-OHDA + L-DOPA + Riluzole vs. 6-OHDA + L-DOPA (Lesion 3 vs Lesion 1)
--> **effect of Riluzole in L-DOPA-treated, lesioned animals**

In the first step of the data analysis we looked at all these type comparisons starting with the most obvious ones focused on identifying differences between the lesioned and the contralateral side of each group. Subsequently, we performed all the other analyses.
In the **transcriptome project**, after looking at all these data, we decided to focus only on the effect of L-DOPA and L-DOPA + Riluzole on gene expression changes.
In the **DNA methylation study**, we performed a more detailed investigation by looking at almost all comparisons to pick out the effect on DNA methylation caused by 6-OHDA injection, L-DOPA administration and Riluzole treatment.

5.3.1 Striatal DA Determination

In order to check the actual presence of dopaminergic denervation as consequence of the unilateral 6-OHDA injection, our collaborator performed an electrochemical detection technique coupled with high-performance liquid chromatography (HPLC-ECD) to assess the dopamine (DA) levels in the ipsilateral (lesioned) and contralateral (control) striatum. As shown in Figure 7, the 6-OHDA lesion results in a drastic decrease (76%) of DA level in the striatum.

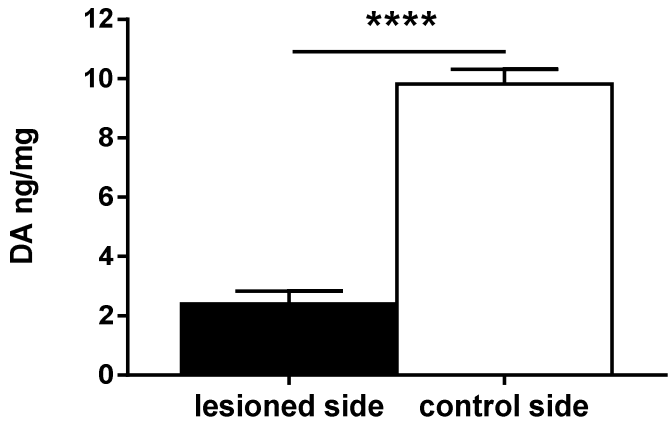

Fig. 7 from [151]

The figure shows the effect of 6-OHDA lesion on the levels of doamine in the striatum.

**** $p < 0.001$

5.3.2 The Triggering of the Phenotype by L-DOPA and Riluzole

The manifestation of locomotor activity as a consequence of the treatment in the rodents can be seen in Figure 8. The chronic administration of L-DOPA led to the appearance of strong abnormal involuntary movements ($p < 0.001$) while the rodent administered with saline only did not show any unusual motor phenotype. Interestingly, the treatment with Riluzole was able to reduce the intensity of AIMs triggered by L-DOPA administration ($p < 0.001$).

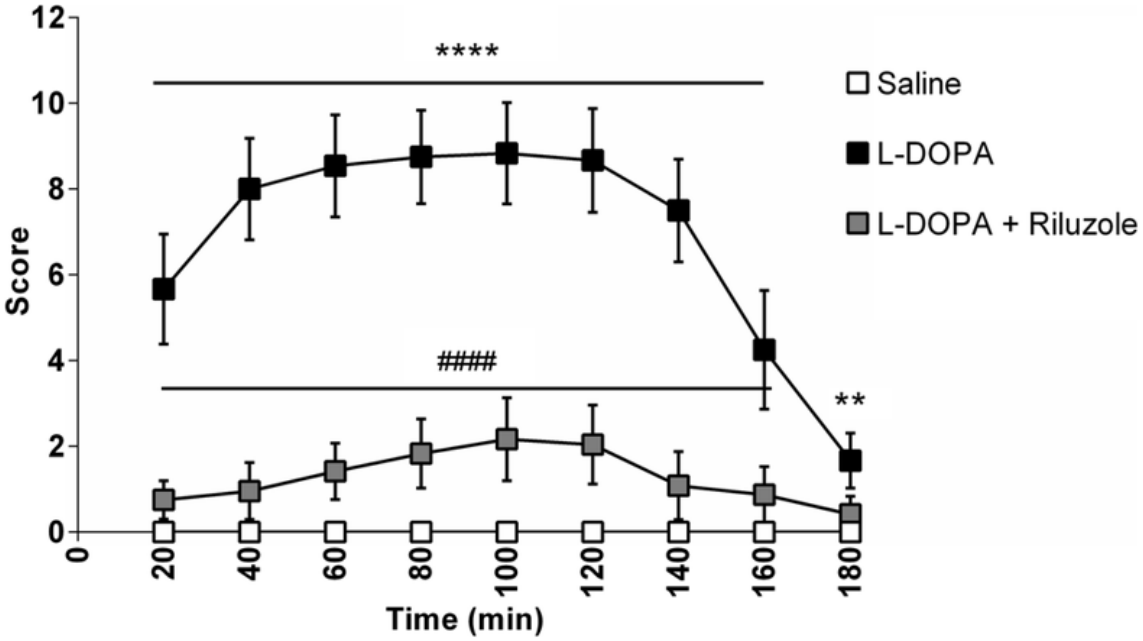

Fig. 8 from [151]

The figure shows the phenotypic observation of the abnormal involuntary movements (AIMs) in our experimental groups. Data is shown as mean ± S.E.M. Significant differences between the saline and L-DOPA-treated groups are indicated as $^{**}p < 0.01$ and $^{****}p < 0.001$ and between the L-DOPA and L-DOPA + Riluzole-treated groups as $^{\#\#\#\#}p < 0.001$

5.3.3 Analysis of the Transcriptome

5.3.3.1 Differentially expressed genes: the mRNA is talking

To identify differentially expressed genes, we fed the RNA data into Ingenuity Pathway Analysis (IPA) bioinformatics tool. Based on the behavioral data (Fig. 8), we decided to study only the effect of Riluzole on gene expression changes and to focus only at the following comparisons: L-DOPA vs. Saline and L-DOPA + Riluzole vs. L-DOPA.

In the first comparison, L-DOPA vs. Saline, we wanted to check the effect of L-DOPA administration on gene expression and we were able to identify 667 unique genes. On the other hand, by looking at L-DOPA + Riluzole vs. L-DOPA we wanted to identify the effect of Riluzole on gene expression and we were able to identify 1200 unique genes (Fig. 9). Furthermore, we found 465 overlapping genes that were differentially expressed

in both comparisons. The hypergeometric distribution test revealed that the overlap of these genes was not random (p = 1.01E-142).

It was surprising to see how all these 465 genes were differentially expressed in the opposite direction: the genes upregulated by chronic L-DOPA treatment were downregulated by Riluzole co-administration and vice versa.

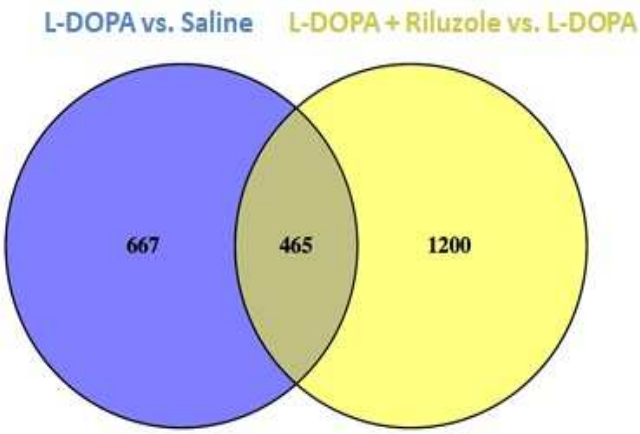

Fig. 9 from [151]

The venn diagram shows the number of significantly differentially expressed genes identified in the two comparisons of interest (FDR corrected p-value < 0.01).

5.3.3.2 Identification of the Top Upstream Regulators

In the L-DOPA vs. Saline analysis (Fig. 9) we identified 667 unique genes that had cAMP-responsive element binding protein 1 (*CREB1*) as top upstream regulator (*CREB1* activation). In L-DOPA + Riluzole vs. L-DOPA analysis we were able to detect 1200 unique genes and, interestingly, they also had *CREB1* as top upstream regulator (*CREB1* inactivation). Moreover, when looking at the overlapping genes (n = 465) *CREB1* came out on top as the upstream regulator. It appears evident that *CREB1* might play a pivotal role in the regulation of locomotor movements. The top 10 upstream regulators of the genes that were differentially expressed in the analysis are listed in Table 12.

Table 12: The top 10 upstream regulators identified in the groups analyzed in our study

Upstream Regulator	Type	Activation z-score	P-value of the overlap	Target genes
L-DOPA vs. Saline				
CREB1	transcription regulator	7.52	8.53E-31	102
Forskolin	chemical toxicant	6.06	5.56E-23	100
Cocaine	chemical drug	5.58	1.02E-19	48
PDGF BB	Complex	5.22	1.74E-19	61
beta-estradiol	chemical - endogenous mammalian	4.05	2.01E-19	178
U0126	chemical - kinase inhibitor	-5.07	7.54E-19	76
CREM	transcription regulator	4.19	7.25E-18	39
dalfampridine	chemical drug	4.58	2.66E-17	21
TGFB1	growth factor	3.82	1.10E-16	166
bicuculline	chemical - endogenous non-mammalian	4.48	7.07E-15	21
L-DOPA + Riluzole vs. L-DOPA				
CREB1	transcription regulator	-6.53	1.21E-11	87
F2	Peptidase	-6.33	8.46E-09	48
TP53	transcription regulator	-2.80	2.41E-08	167
CREM	transcription regulator	-2.73	3.16E-08	32
2-amino-5-phosphonovaleric acid	chemical - other	3.20	3.57E-08	31
PDGF BB	Complex	-5.31	6.20E-08	52
Forskolin	chemical toxicant	-5.48	1.26E-07	87
HIF1A	transcription regulator	-4.27	2.98E-07	58
CD40LG	Cytokine	-2.50	3.17E-07	57
U0126	chemical - kinase inhibitor	4.82	4.30E-07	67
Overlapping genes (FC for L-DOPA + Riluzole vs. L-DOPA)				

CREB1	transcription regulator	-6.19	1.17E-24	58
Forskolin	chemical toxicant	-5.88	5.08E-22	60
Cocaine	chemical drug	-4.32	1.71E-15	28
CREM	transcription regulator	-3.00	5.60E-15	24
dalfampridine	chemical drug	-3.74	2.92E-14	14
PDGF BB	Complex	-4.98	3.68E-14	33
U0126	chemical - kinase inhibitor	4.57	4.27E-13	39
kainic acid	chemical toxicant	-4.33	7.23E-13	22
bicuculline	chemical - endogenous non-mammalian	-3.65	8.91E-13	14
IL1B	Cytokine	-3.60	1.59E-12	54

Table 12 from [151]

The table describe the top 10 upstream regulator of the comparison L-DOPA vs. Saline, the comparison L-DOPA + Riluzole vs. L-DOPA and the overlap between the two groups.

5.3.3.3 CREB1 target genes

Based on all the upstream regulator results, we decided to focus only on *CREB1*. *CREB1* encodes for a transcription factor that modulates the expression of many target genes involved in neuronal processes such as neuronal survival, differentiation and development. The RNA data revealed that *CREB1* targets genes identified in the transcriptome analysis are regulated in the opposite direction by L-DOPA and Riluzole (Table 13).

Table 13: The fold change of CREB1 target genes

Gene	Gene name	L1vsL2	L3vsL1
SRXN1	sulfiredoxin 1	6.08	-3.28
SLC32A1	solute carrier family 32 member 1	1.79	-1.52
Sik1	salt inducible kinase 1	3.10	-2.08
SERTAD1	SERTA domain containing 1	2.83	-1.74
SCG2	secretogranin II	2.90	-1.69

RHEB	Ras homolog enriched in brain	1.49	-1.18
REM2	RRAD and GEM like GTPase 2	5.42	-2.82
Pvr	poliovirus receptor	2.84	-2.34
PTGS2	prostaglandin-endoperoxide synthase 2	5.15	-3.08
PER1	period circadian clock 1	2.18	-1.87
PDYN	Prodynorphin	2.50	-1.75
PDXK	pyridoxal kinase	1.93	-1.69
NR4A3	nuclear receptor subfamily 4 group A member 3	10.26	-4.16
NR4A1	nuclear receptor subfamily 4 group A member 1	4.27	-2.15
NPTX2	neuronal pentraxin 2	4.96	-2.70
NPAS4	neuronal PAS domain protein 4	5.50	-5.07
NFIL3	nuclear factor, interleukin 3 regulated	2.08	-1.51
MIDN	Midnolin	2.52	-2.34
JUNB	JunB proto-oncogene, AP-1 transcription factor subunit	7.83	-3.28
IRS2	insulin receptor substrate 2	6.01	-3.00
INHBA	inhibin beta A subunit	5.76	-3.41
IGSF9B	immunoglobulin superfamily member 9B	1.93	-1.63
GPR3	G protein-coupled receptor 3	8.19	-3.95
GADD45G	growth arrest and DNA damage inducible gamma	4.59	-2.92
GADD45B	growth arrest and DNA damage inducible beta	3.43	-1.76
GAA	glucosidase alpha, acid	1.46	-1.34
FRMD6	FERM domain containing 6	2.16	-1.59
FOSB	FosB proto-oncogene, AP-1 transcription factor subunit	17.17	-4.96
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	12.99	-4.94
FGF13	fibroblast growth factor 13	-1.29	1.26
EHD4	EH domain containing 4	1.30	-1.30
EGR4	early growth response 4	10.01	-4.16
DUSP14	dual specificity phosphatase 14	4.15	-2.44

CSRNP1	cysteine and serine rich nuclear protein 1	3.06	-1.88
CDKN1A	cyclin dependent kinase inhibitor 1A	4.09	-2.57
BAG3	BCL2 associated athanogene 3	1.80	-1.73
ATF3	activating transcription factor 3	10.98	-6.06
ARC	activity regulated cytoskeleton associated protein	6.41	-2.99
CREM	cAMP responsive element modulator	1.73	-1.52
JUN	Jun proto-oncogene, AP-1 transcription factor subunit	2.45	-1.70
PIM3	Pim-3 proto-oncogene, serine/threonine kinase	1.41	-1.39
VEGFA	vascular endothelial growth factor A	1.33	-1.30
TAC1	tachykinin precursor 1	2.82	-1.92
STAT3	signal transducer and activator of transcription 3	1.13	-1.13
SH3KBP1	SH3 domain containing kinase binding protein 1	-1.15	1.13
PLAT	plasminogen activator, tissue type	1.47	-1.48
NPY	neuropeptide Y	1.72	-2.23
KLF4	Kruppel like factor 4	2.00	-1.60
HSPA5	heat shock protein family A (Hsp70) member 5	1.64	-1.41
CRH	corticotropin releasing hormone	6.32	-4.62
CDK19	cyclin dependent kinase 19	-1.22	1.18
SEMA7A	semaphorin 7A (John Milton Hagen blood group)	1.30	-1.27
Nrgn	Neurogranin	1.56	-1.36
LMO1	LIM domain only 1	1.25	-1.23
CCND3	cyclin D3	1.22	-1.31
CDC37	cell division cycle 37	1.27	-1.28
ID1	inhibitor of DNA binding 1, HLH protein	1.44	-1.57
NDUFV1	NADH:ubiquinone oxidoreductase core subunit V1	1.19	-1.21

Table 13 from [151]

The table reports the fold change of the CREB1 target genes differentially expressed in L-DOPA vs. Saline (L1vsL2) and L-DOPA + Riluzole vs. L-DOPA (L3vsL1) (FDR-corrected p value < 0.01).

5.3.3.4 Revealing the molecular mechanism of Riluzole

To build a molecular mechanism of action of Riluzole, we looked only at CREB1 as the top upstream regulator of the differentially expressed genes. We performed literature research to look to the functions and all the possible interactions of the proteins encoded by CREB1 target genes (Table 13). First, we used UniProt [154] to check the function and localization of the genes and their encoded proteins. Subsequently, we used PubMed (http://www.ncbi.nlm.nih.gov/pubmed) to search for all the experimentally validated interactions between the proteins as well as their involvement in L-DOPA, Riluzole or Parkinson's Disorder (PD). Lastly, we built a molecular landscape depicting all those protein interactions (Fig. 10).

The extensive description of all the protein interactions happening in the striatal neuron are described below. The encoded by these genes are indicated in bold and capital letters.

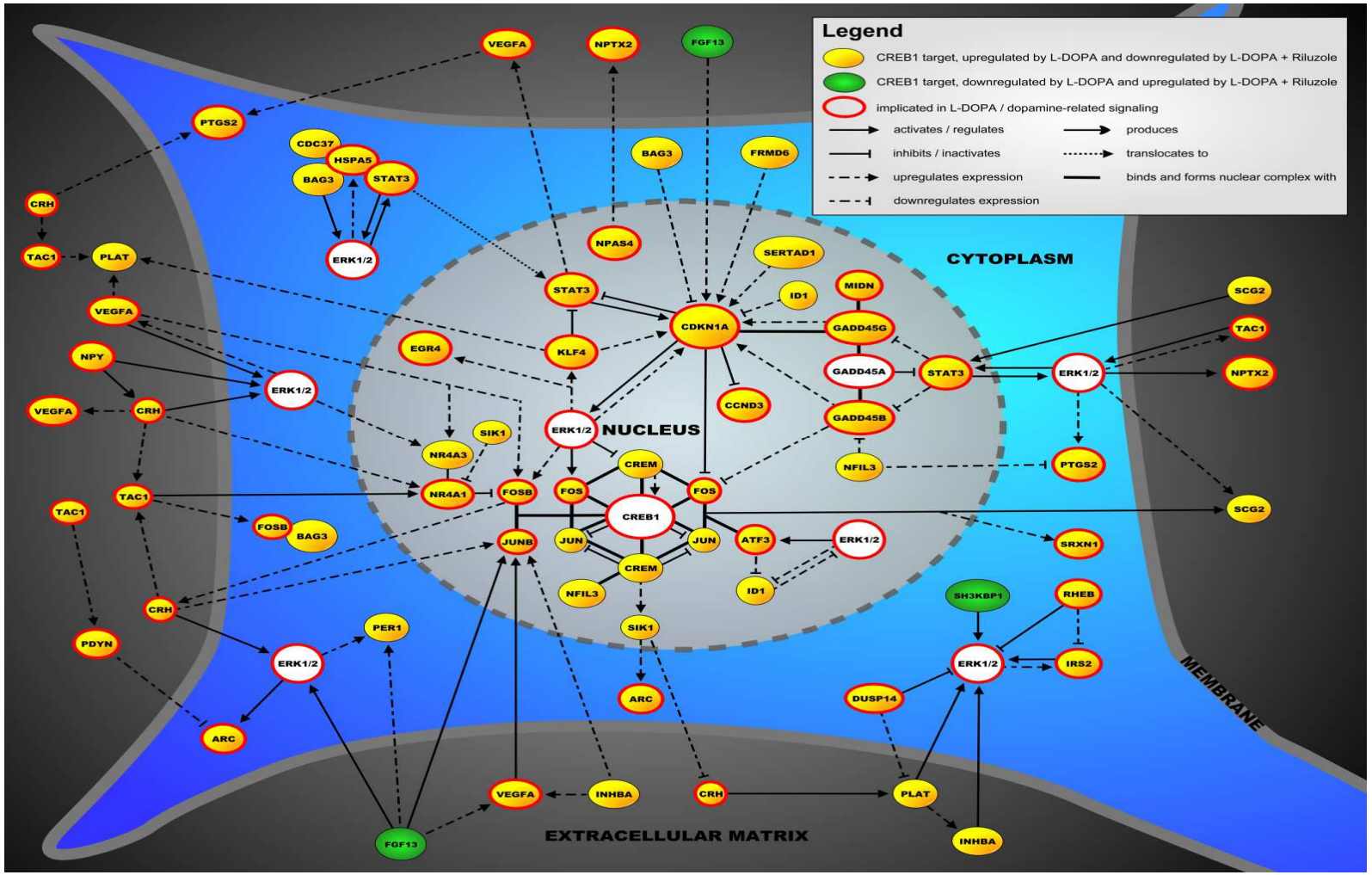

Fig. 10 from [151] The molecular landscape is located in a neuron and shows the functional interactions between proteins encoded by 43 of the 58 *CREB1* target genes regulated in the opposite direction by L-DOPA and after Riluzole co-administration in our AIMs model.

The molecular landscape is gathered around CREB1 localized in the striatal neuron. As key signaling 'hub' in the landscape, CREB1 is a pivotal transcription factor that modulates the expression of many genes implicated in neuronal activities such as neuronal survival, development and differentiation. The literature data have revealed that, at least in striatal neurons, CREB1 transcriptional function is activated by L-DOPA [155, 156]. Moreover, **CREM** (a component of the CREB transcription factor family) connects to CREB1 and regulates its expression. The complex composed of CREB1-**CREM** connects also with **FOS** and **JUN** [157]. **FOS** and **JUN** connect to each other as well as with **FOSB** and **JUNB**. **FOS**, **JUN**, **FOSB** and **JUNB** are part of the transcriptional factor complex called AP-1 which engages with CREB1 [158-162] and is known to regulate the apoptotic process in response to stress as well as DNA damaging agents [163]. Furthermore, **JUN** transcriptional activity is inhibited by both CREB1 and **CREM** [157]. Also, **FOS** and **JUN** connect with **ATF3** [164] and **CREM** engages with the transcription factor **NFIL3** [165] (see below). **JUN** modulates the differentiation of neurons and the survival of cells. In particular, **JUN** is able to defend neuronal cells (PC12 cells) from the apoptotic process [166]. Interestingly, higher expression levels of **FOS** were detected in rodents that underwent 6-OHDA injection [167, 168] followed by L-DOPA administration [169-171].The treatment with L-DOPA results in a higher expression level of ***Fosb*** but not ***Junb*** [172]. In line with these evidences, ***Junb*** was found to be a protective agent against nigral neurons cell death [173].

Additionally, the extracellular signal-regulated kinases 1 and 2 (ERK1/2) - which can be located in the cytoplasm and the nucleus – switch on CREB1 and **CREM** (not shown) [174, 175]. Not to mention, ERK1/2 are implicated in higher **FOSB** expression [176] and switch on **FOS** [177, 178] as well as **ATF3** [179]. ERK1/2 were classified as kinases modulating the survival of neurons but it has been shown that ERK1/2 also regulate the secondary damage mechanisms involved in many neurodegenerative disorders, stroke, injury and autoimmune diseases of the CNS [180-185]. Furthermore, several studies showed that L-DOPA administration activates ERK1/2 [139, 186-188]. **ATF3** and ERK1/2 negatively modulate the expression of the anti-apoptotic transcription factor **ID1** [189-191], which in turn decreases the expression of ERK1/2 [192]. The expression of **ATF3** is usually very low in both neurons and glia cells but rises in response to injury or stressful stimuli, thus leading to a positive regulation of cell survival [193]. In line with

our findings, a chromatin immunoprecipitation study revealed that acute L-DOPA treatment in 6-OHDA-lesioned mice increase the expression of ***Atf3*** as well as the transcription factors ***Klf4*** and ***Npas4*** transcription factors (see below) [194].

Moreover, **NR4A1** and **NR4A3** are part of the NR4A orphan nuclear receptor transcription factor family which is upregulated under stressful stimuli in the CNS. This family of transcription factors (NR4A) is pivotal in the survival of neurons downstream of CREB (1) signaling and they might represent a good drug target in neurodegenerative disorders [195].

In the MPTP mouse model of PD, the activation of **Nr4a1** results in the preservation of dopaminergic neurons [196]. Additionally, ***Nr4a1*** knockout mice show increased locomotor activity, higher sensitivity to dopamine [197] and stronger rotational behavior induced by L-DOPA [198]. Furthermore, the administration of L-DOPA in MPTP monkeys (a non-human primate model of PD) results in increased expression of ***Nr4a1*** is increased in the upon L-DOPA treatment in MPTP monkeys [199]. Not to mention, analyses on blood on PD patients detected reduced ***Nr4a1*** expression [200]. All these findings corroborate our results revealing **NR4A1** overexpression. In the molecular landscape, **NR4A1** connects with **NR4A3** resulting in a functional complex [201], **NR4A1** reduces **FOSB** activity [202] while **SIK1** kinase is upregulated by **CREM** [203] – reduces the expression of **NR4A1** [204]. On top of that, **NR4A1** is activated by **TAC1** [205], an extracellular anti-apoptotic protein with extracellular localization [206] highly expressed in a primate model of L-DOPA induced dyskinesia [207]. **TAC1** also increases the expression of **FOSB** [208], which can also be located in the cytoplasm where it constitutes a molecular complex with **BAG3**, a co-chaperone protein involved in the regulation of neuronal apoptosis and autophagy [209, 210]. Noteworthy, the autophagy modulated by ***Bag3*** is implicated in the clearance of aggregated proteins often linked to neurodegenerative disorders [211-213]. To conclude, the expression of **JUNB** [214] is upregulated by the corticotropin-releasing hormone (**CRH**), a central nervous system (CNS) neurotransmitter [215] involved in the regulation of neuronal apoptosis [216] as well as the activity of dopaminergic neurons [217]. The expression of **CRH** is increased by **FOSB** [218]. The expression of **NR4A1** [219], **TAC1** [220] and the growth factor **VEGFA** (see below) [221] is positively regulated by **CRH** which switches also on ERK1/2 [222]. On the other hand, ERK1/2 positively modulate **NR4A3** [223].

Another major cascade depicted in the molecular landscape originates from **CDKN1A**, a transcription factor implicated in the death of dopaminergic cells as consequence of 6-OHDA injection [224]. **CDKN1A** turns off both **FOS** and **JUN** [225] while it switches on ERK1/2 [226]. The expression of **CDKN1A** is increased by **KLF4** and ERK1/2 [227] and the expression of **KLF4** is upregulated by ERK1/2 [228]. In addition, the expression of **CDKN1A** is increased by the growth factor **FGF13** (see below), the transcription factor **SERTAD1** and the cytoplasmic protein **FRMD6** [229-231] whereas the expression of **CDKN1A** is reduced by **ID1** and **BAG3** [232, 233]. **CDKN1A** turns also off **CCND3** [234], a cell cycle regulator which is highly expressed during the apoptosis of dopaminergic neurons induced by 6-OHDA injection. It is a very interesting evidence considering that a dysregulation of cell cycle has been proposed as one of the causes leading to the death of dopaminergic neurons in Parkinson's disease (PD) [235]. In addition, **GADD45G** – a member of the GADD45 family whit pivotal role in the regulation of apoptosis [236] – connects and engages with **CDKN1A** thus leading to its increased expression [237]. **GADD45G** connects and engages also with **MIDN** [238], a gene strongly linked to PD [239, 240]. **GADD45G**, **GADD45A** and **GADD45B** – which also positively regulates the expression of **CDKN1A** [241] – connects with each other to generate a molecular complex [242]. Noteworthy, the knockout of **Gadd45b** in L-DOPA-induced dyskinesia (LID)-rodent model leads to higher expression of **FOS** and **FOSB** [243], which hints that ***Fos*** and ***Fosb*** expression is downregulated by **GADD45B** (not shown). A second study on LID rodent model reported the treatment with L-DOPA increased ***Gadd45b*** and ***Gadd45g*** expression in the lesioned side of the striatum [244]. ***Gadd45a*** is also implicated in 6-OHDA-induced brain toxicity [245]. At last, ***Gadd45b*** expression is downregulated by **NFIL3** [246], a transcription factor with neuronal neuroprotective function [247].

Several of the proteins depicted in the molecular landscape engage with **STAT3**, a protein that promotes the proliferation of neuron to contrast the cell death induced by 6-OHDA injection [248]. In the nucleus, **STAT3** is turned off by **GADD45A** [249], **KLF4** [249] and **CDKN1A** [250]. On the other hand, **STAT3** turns on **CDKN1A** [251] and negatively modulates **GADD45B** and **GADD45G** expression [252, 253]. In the cytoplasm, **STAT3** engages with **HSPA5** to originate a molecular complex. As shown in the landscape, **CDC37** and **BAG3** bind and interact with this complex [254, 255]. Worth

to mention, a rodent model of PD has shown that high levels of ***Hspa5*** are able to defend dopaminergic neurons from degeneration [256]. **HSPA5** expression is upregulated by ERK1/2 [257]. In turn, **BAG3** negatively modulates ERK1/2 activity [258]. To complicate things, **STAT3** and ERK1/2 positively regulate each other [259, 260]. Furthermore, the expression of **VEGFA** is upregulated by **STAT3** (see below) which is activated by the extracellular neuroendocrine protein secretogranin-2 (**SCG2**) [259]. **SCG2** encodes for a protein called secretoneurin (SN) (not shown) which is involved in differentiation of neurons [261] and is also able to increase the expression of anti-apoptotic proteins via **STAT3**-dependent cascade [259]. The expression of **SCG2** is regulated by ERK1/2 [262] while **FOS**-**JUN** modulate the activity of **SCG2** [261]. **FOS**-**JUN** are also involved in the positive modulation of **SRXN1** [263], a cytoplasmic enzyme with a protective role in the deterioration of dopaminergic neurons observed during oxidative stress [264, 265].

In the molecular landscape, many signaling cascades have **ERK1/2** as main actor. To start with, ERK1/2 is activated by **TAC1** (see above) [266], while the expression of **TAC1** [267] and **PTGS2** [268] is upregulated by ERK1/2. The regulation of the cytoplasmic enzymes encoded by **PTGS2** is controlled positively by **CRH** [269] and **VEGFA** [270] (see above) and negatively by **NFIL3** (see above) [271]. Further, the activity of **NPTX2** is regulated by ERK1/2 [133, 272], while NPAS4 upregulates the expression of **NPTX2** expression [273]. **NPTX2** plays a role in the development of neurons, the formation of synapses, and the growth of neurites [274, 275]. Moreover, one study on PD patients observed, in the substantia nigra, a really high upregulation (>800%) of **Nptx2** [275] while another study showed that L-DOPA treatment increases the expression of ***Nptx2*** and the severity of locomotor activity decreases in ***Nptx2*** knockout mice [133]. In addition to this complicated cascade, the **SH3KBP1** and **IRS2** regulate the activity of ERK1/2 activity [276]. **IRS2** encodes for cytoplasmic protein, usually involved in the apoptotic process, positively modulated by ERK1/2 [277]. Worth to mention, the injection of 6-OHDA results into **Irs2** degradation [278]. Moreover, the expression of IRS is decreased by the GTP-binding protein **RHEB** [279] which is also able to turn off ERK1/2 [280]. **RHEB** encodes for a gene that is involved in the regulation of neuronal plasticity and differentiation. One study in an animal model of PD reported that **RHEB** is needed to conserve and repair the striatal dopaminergic axons [281-283].

Interestingly, if overexpressed, ***Rheb***'s function switches and it results in the activation of the apoptosis process. The expression of **EGR4** is also upregulated by ERK1/2. **EGR4** encodes for a transcription factor that is increased as consequence of L-DOPA administration [133]. A negative regulator of ERK1/2 is represented by **DUSP14** [284, 285]. Striatal neurons lacking dopamine in LID model showed downregulation of the ERK signaling [138, 286]. **DUSP14** is also responsible for the reduced expression of the extracellular enzyme **PLAT** [287] which is involved in migration and plasticity of neurons [288]. The pro-survival/anti-apoptotic role of **Plat** has been shown by in-vitro and ex-vivo studies [289]. The expression **INHBA** is increased by **PLAT** [290] while the activation of ERK1/2 is regulated by **PLAT** and **INHBA** [291, 292].

Moreover, **INHBA** positively modulates **JUNB** [293] and **VEGFA** expression [292]. In turn, the activation of **JUNB** [294] and ERK1/2 [295] is modulated by the growth factor **VEGFA** which is positively regulated by the growth factor **FGF13** [296]. **FGF13** modulates the activity of **JUNB** [158]. The administration of **VEGFA** to 6-OHDA-lesioned rats results into a significant boost of neurons [297]. To continue, the expression of **FOSB** and **NR4A3** is increased by **VEGFA** [298, 299] (see above) while the expression of **PLAT** is increased by **VEGFA**, **TAC1** and **KLF4** [300-302]. **PLAT** is also activated by **CRH** [303] while the activation of **CRH** is regulated by **NPY**, an extracellular hormone redundant in the CNS where it modulates the apoptotic process in a negative way and it controls the autophagic process in a positive way [304, 305]. The activation of ERK1/2 is regulated also **NPY** [306]. A study on PD patients showed increased striatal expression of the neuroprotective agent **NPY** [307]. Further, **SIK1** downregulates the expression of **CRH** [308] (see above). The expression of **ARC** is upregulated by **SIK1**. **ARC** encodes for a brain-specific protein involved in the apoptotic process [309] that was found upregulated in LID striatal neurons [310, 311]. Further, the activation of **ARC** is modulated ERK1/2 [312] while **PDYN** turns off **ARC** [313]. **PDYN** encodes for a neuropeptide with anti-apoptotic function [314]. The expression of **Pdyn** is upregulated by **TAC1** [208]. Once again, LID animal model studies detected increased striatal **PDYN** expression [315-317]. To conclude, the expression of **PER1** is upregulated by ERK1/2 [318] and **FGF13** [319] while it is blocked by **NFIL3** [154] (not shown). The activation of ERK1/2 is modulated also by **FGF13** [320].

5.3.3.5 Validation of the mRNA data

In order to validate the RNA sequencing data, we performed a qPCR on 10 CREB1 target genes. As shown in Fig. 11, we were able to validate 8 out of 10 genes therefore we assumed that the RNA sequencing data used in this project reflect real expression changes.

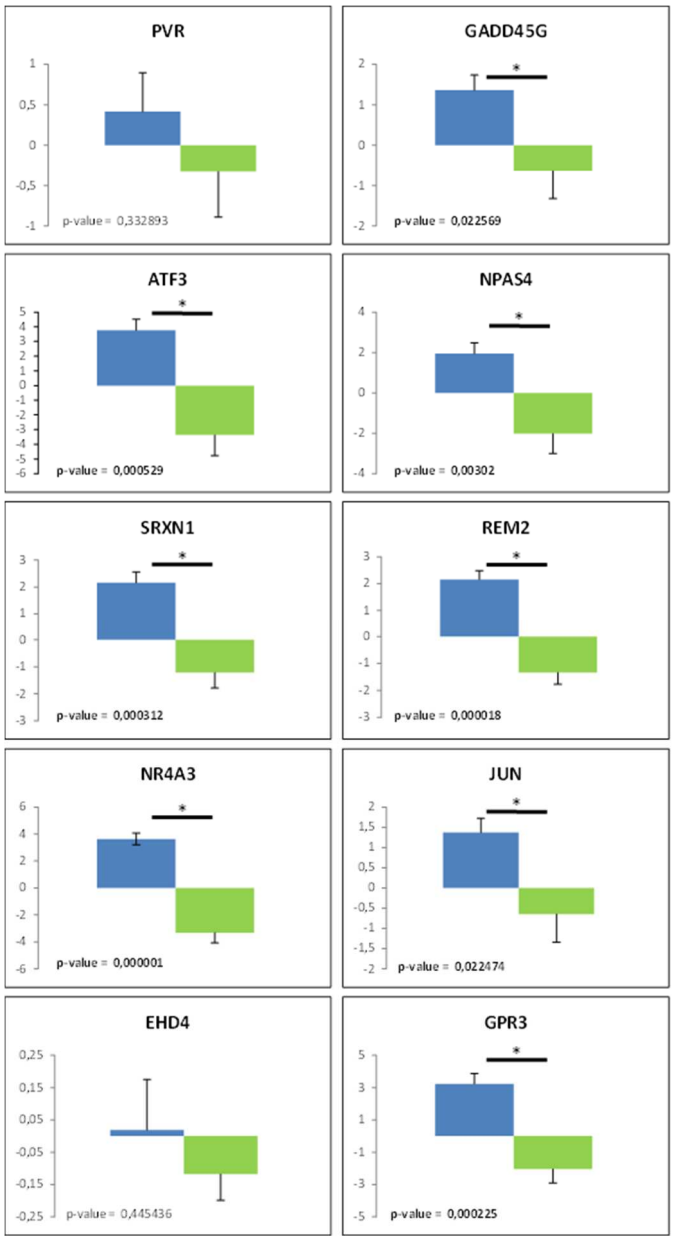

Fig. 11 from [151]

The figure shows the qPCR values [log_2FC (Mean + SEM)] of the genes selected for the validation of mRNA data, blue represents L-DOPA vs. Saline while green represents Saline and L-DOPA + Riluzole vs. L-DOPA.

5.3.4 DNA methylation profile in the striatum of our AIMs model

In order to study the methylation pattern in the striatum of rodents showing abnormal involuntary movement we decided to perform genome wide methylation studies (RRBS). As shown in Table 11, we had 3 experimental groups that underwent different treatment. To extrapolate the effect of each treatment on methylation levels, we firstly compared samples within the same group (lesion and contralateral side) and later on between different groups using a similar approach:

1st step -> comparison of the two datasets to identify the common CpGs
2nd step -> observation of the genomic distribution of the identified CpGs
3rd step -> identification of differentially methylated sites (DMSs)
4th step -> annotation and gene enrichment analysis

5.3.4.1 Distribution of CpGs in the rat genome

In case of the DNA methylation experiment (n = 3), we did not have the same statistical power as for the RNA sequencing (n = 8), therefore we took a different approach and started by comparing the lesion to the control side. In each group analyzed (3 animals, control and lesioned sides) the RRBS protocol was able to detect approximately 435,000 CpGs. The majority of CpGs (>50%) was located in intergenic regions (Fig. 12). In promoters and introns, we detected approximately an equal amount (~20% each). The remaining CpGs were located in exons (less than 10%). The distribution of CpGs in the rat genome was similar for all groups investigated in this study (Group 1, Group 2 and Group 3 see Table 11).

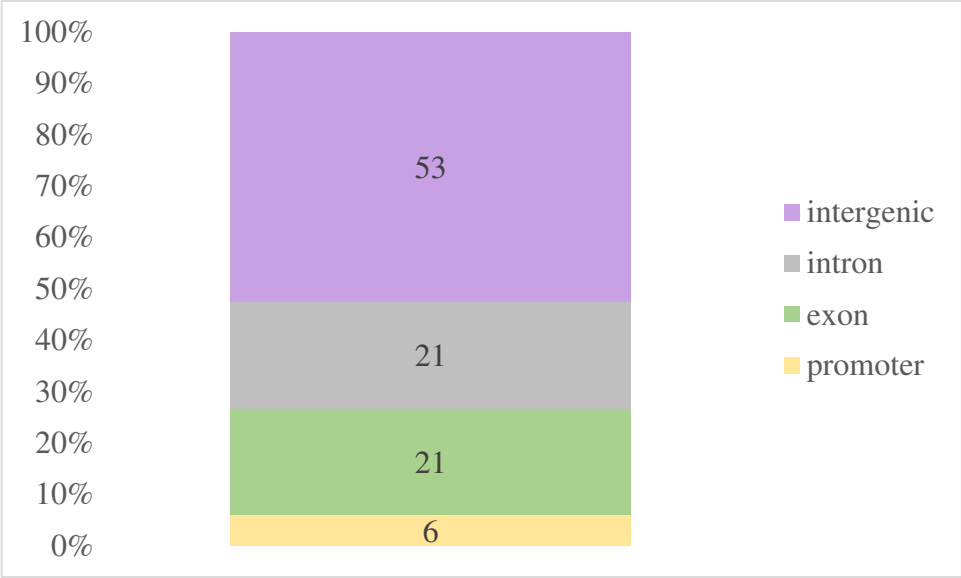

Fig. 12: The figure shows the distribution of CpGs (%) in the rat genome.

We also looked at the distribution of CpGs in the area of the genome enriched in cytosines (Fig. 13). The most CpG dense regions are called CpG islands (CGI), while area of the genome with intermediate CpG density, located within 2kb of CpG islands, are called CpG shores. The majority of the CpGs (>60%) were located outside islands and shores. In CpG islands, we found more than 20% of the targeted CpGs while the remaining CpGs are located in shores (10%). The distributions of cytosines identified and analyzed was similar for all groups investigated in this study.

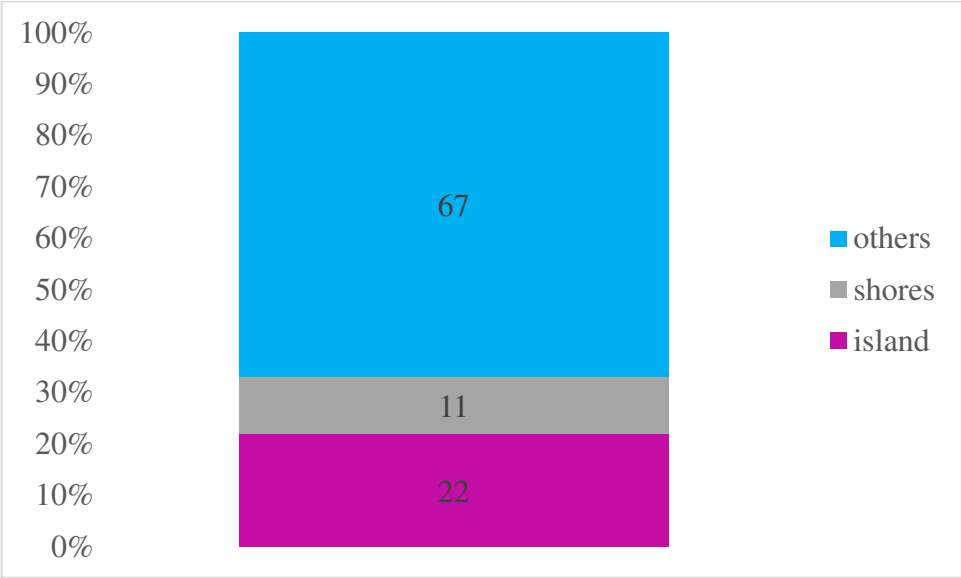

Fig. 13: The figure shows the distribution of CpGs (%) in CpG enriched area.

5.3.4.2 Identification and localization of the methylated sites

To identify the effect of 6-OHDA injection alone or in combination with either L-DOPA administration or the impact of Riluzole treatment, we looked at each individual group and compared the methylation levels of control sides to the methylation levels of the lesioned side to identify the presence of differentially methylated sites (DMSs). DMSs were designated as CpG dinucleotides with statistically significant methylation level differences after correction for multiple testing ($q < 0.01$). Table 14 shows the total number of striatal DMSs. The highest number of DMSs (54) were detected in the lesion only group (G2) and distributed almost equally (25, 29) in decreased (hypo) and increased (hyper) methylation levels. In the case of the L-DOPA treated group (G1) we detected only 14 DMSs, all of them undergoing loss of methylation, while the administration of both L-DOPA and Riluzole treatment (G3) resulted in very few DMSs.

Table 14: Number of DMSs detected in each group when comparing the lesion to the contralateral side of striatum

	Hyper	Hypo	Total
G1	0	14	14
G2	25	29	54
G3	4	1	5

All together these numbers indicate that less than 0.02% of all investigated CpGs underwent statistically significant DNA methylation changes. These findings show that methylation levels in the lesioned and the contralateral striatum are very similar regardless the type of treatment administered.

The behavioral data (Fig. 8) showed that 6-OHDA injection alone has no effect on the locomotor activity of the animals, while the methylation data revealed almost no changes. As next step, we decided to compare the DNA methylation levels between different groups. We first compared the control side ($n = 3$) of the L-DOPA treated group (G1) with the control side of the animals undergoing no treatment (G2). As indicated in the Table 15, we identified 1125 DMSs. Very similar results were observed when we

compared the lesioned side. Interestingly, when we looked at the control side of the L-DOPA + Riluzole treated animals (G3 group) and the control only animals (G2) we identified even more DMS, the majority undergoing hypomethylation (Table 15). Surprisingly, when we looked at the L-DOPA (G1) and the L-DOPA + Riluzole (G3) comparison, we detected very few DMSs (only slightly superior in the number of the comparisons described in the Table 14).

Table 15: Number of DMSs identified when comparing different groups

	Hypo	Hyper	Total
G1 vs. G2 Control side	867	258	1125
G1 vs. G2 Lesion side	687	253	940
G1 vs. G3 Control side	54	64	118
G1 vs. G3 Lesion side	46	67	113
G3 vs. G2 Control side	1260	256	1516
G3 vs. G2 Lesion side	1143	289	1432

By looking at the lesioned and control side it is impossible to observe any significant differences in the number of hypomethylated and hypermethylated DMSs. Altogether these data further confirm that the lesioned and contralateral side of the striatum have almost identical methylation profiles. Furthermore, they also strengthen our hypothesis that 6-OHDA treatment induces only very few DNA methylation changes while the systemic drug treatments have similar effect on both side of the striatum.

5.3.4.3 A new way of analysis: merging the lesion and contralateral side

Based on the high similarity of the methylation levels observed so far, we decided to merge the 3 lesioned and the 3 control sides of each group to obtain a new group made up of the 6 striata in order to increase the statistical power. Moreover, we also relaxed the bioinformatic analysis criteria: the detection of a CpG in at least 4 out of the 6 striata was mandatory for any CpG to be included in the analysis. By doing so, we could substantially increase the number of analyzed CpGs (~600,000) without altering the genome-wide distributions of this background.

Subsequently, we looked at the presence of DMSs, and we observed a global increased number of significantly methylated sites compared to the previous analysis where lesioned and control sides were separately compared (Table 16). The overall higher number of hypomethylated CpGs of the L-DOPA treated animals was still prevalent. Indeed, the comparison G1 vs. G2 picked-up 1243 hypomethylated sites while the comparison G3 vs. G2 detected 2106 hypomethylated sites. Moreover, the comparison G1 vs. G3 (Table 16) detected very few DMSs (297) without a clear tendency for either hypo- or hypermethylation. Based on these results we decided to focus our attention only on the G1 vs. G2 and G3 vs. G2 comparisons.

Table 16: Number of DMSs after merging the lesion and the contralateral side in a single group

	Hypo	hyper	Total
G1 vs. G2	1243	510	1753
G1 vs. G3	179	118	297
G3 vs. G2	2106	530	2636

The genomic distribution of the DMSs (Fig. 14) revealed that most of the significantly methylated sites are located in intergenic regions (~60%) or intronic area (~25%) while a small portion can be located in promoters (~6%).

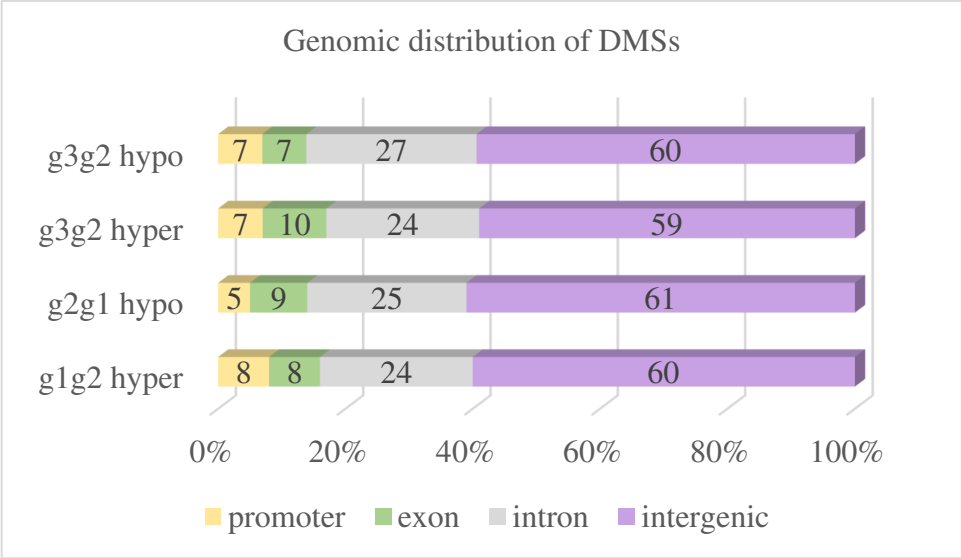

Fig.14: The figure shows the distribution of DMSs (%) in the rat genome.

The distribution of DMSs in CG rich area showed that the majority of DMSs detected by the analysis are located outside CpG islands or shores (85%) (Fig. 15). Once again, we did not see any significant difference in the distribution of CpGs between the different groups analyzed.

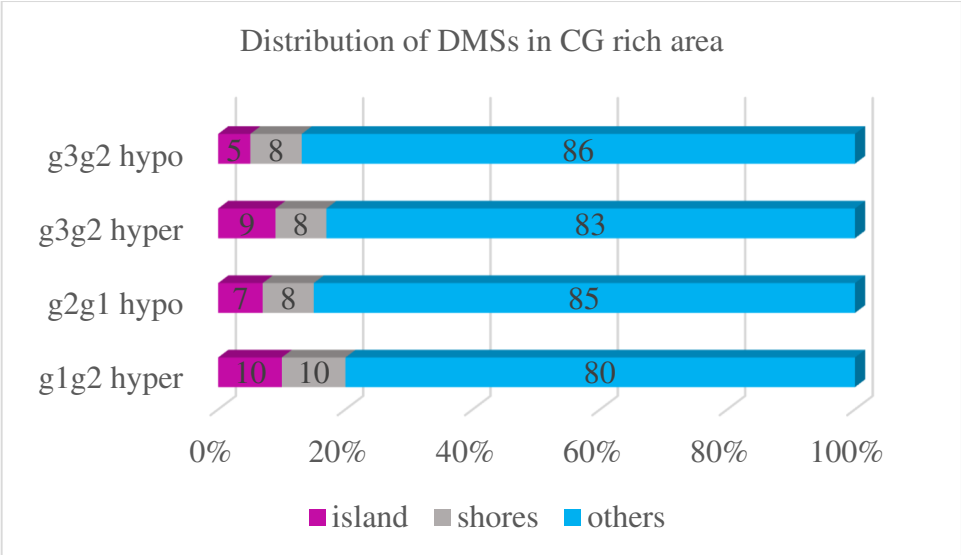

Fig.15: The figure shows the distribution of DMSs (%) in CG enriched area.

5.3.4.4 Genes with marked changes in methylation

To further analyze G1 vs. G2 and G3 vs. G2, we looked at the density of DMSs to check if the similarities observed so far were systemic. To check the density, we built "segments" of 1Mb and counted the number of DMSs detected in each segment for both comparisons.

The subtraction of these segments (X segment (G1 vs. G2) – Y segment (G3 vs. G2)) revealed which were the segments with the biggest difference in number of DMSs. The density analysis revealed that there are some genomic regions which differ a lot between the two comparisons. Moreover, the combined treatment of L-DOPA and Riluzole resulted in the identification of some interesting segment. For example, the region nearby the phosphatidylinositol glycan anchor biosynthesis class X (*PIGX*) gene, had 9 DMSs in L-DOPA + Riluzole treatment but none in L-DOPA only administration. The physiological function of this gene is involved in the biosynthesis of glycolipids. Similarly, the region nearby the COMM domain containing protein 1 (*COMMD1*) does show 6 DMSs in case of L-DOPA + Riluzole treatment. *COMMD1* encodes for a scaffold protein involved in the modulation of the aggregation of misfolded protein typically observed in PD. However, the data revealed that treatment with L-DOPA only or with L-DOPA + Riluzole results in a similar methylation profile, with the exception of these few genomic areas. Indeed, we observed a clear correlation between the methylation level of CpGs in the animals (r = 0.52). This correlation became even higher (r = 0.98) by looking at DMSs in treated groups (L-DOPA and L-DOPA + Riluzole) relative to the saline only group.

In the next step, we decided to look at those genes which underwent marked changes in DNA methylation levels upon the administration of L-DOPA or L-DOPA + Riluzole (Table 18).

Table 18: Number of DMSs with drastic changes in methylation levels

	L-DOPA	L-DOPA + Riluzole
=>80% methylation levels	14	22
<=80% methylation levels	68	66

We first looked at the changes of at least 80% in either loss or gain of methylation. The treatment with L-DOPA resulted in 68 DMSs exhibiting at least 80% loss of methylation and 14 DMSs having at least 80% increased of methylation. On the contrary, the L-DOPA + Riluzole treatment resulted in 66 DMSs exhibiting at least 80% loss of methylation and 22 DMSs having at least 80% increased of methylation. Table 19 shows the genes that we were able to annotate using these DMSs. In bold are listed the genes with literature reports with terms "L-DOPA" or "Brain". Once again, we observe many similarities in the DMSs detected in the two groups.

Table 19: List of the genes showing drastic changes in methylation levels

	<=80% methylation levels	=>80% methylation levels
L-DOPA	*AlzDis, Cox6b1, Ctnnbl1* *Dcdc2,* ***Itih4****, Kif5a* *Ly6e,* ***Mtm1****, Nudt16* *Ppp1r13b,* ***Robo1***	*Fndc3b,* ***Mad1l1***
L-DOPA/ L-DOPA + Riluzole common genes	*Akna, Dlgap2, Masp1* ***Morn4****, Myh7b, Nid2* *Padi3, Plcg2, Tgm2* *Ttll1, Zfand3, Zfyve21*	***Asic2****, Dnai2, Ntng1*
L-DOPA + Riluzole	***Disc1****, Fer1l4, Irf2* *Paxip1, Pip5k1b, Plekha5* *Ppp1r13b, Slc16a10,* *Ush2a,* *NM_001107843,*	*Hip1*

5.3.4.5 The biological relevance of the DMSs

To have a clear picture of the biological role of the DMSs picked-up by RRBS, we performed a gene enrichment analysis using the GOrilla tool. In these analyses we compared any DMSs (either hypo or hyper) located nearby genes to its relative background. The background was defined as all the genes in the dataset that had at least one DMS detected.

Interestingly, the G1 vs. G2 comparison identified 549 genes, and the enriched GO terms analysis showed many terms directly related to processes like neurodevelopment, neurotransmission, glutamate signaling, and neuron remodeling. The G3 vs. G2 comparison yielded similar results: we identified 830 genes and the GO term analysis showed terms related to synapse function, neuron differentiation, epigenetic modifications, dendrite development and glutamatergic neurotransmission.

6. DISCUSSION

6.1 Genetic studies of Tourette Syndrome

In the genetic association studies, we wanted to investigate SNPs in the 3'UTR of Tourette candidate genes. We decided to focus mostly on genetic variations predicted to alter the binding site of miRNAs. Indeed, there is a chance that SNPs in that area might have a greater impact on the gene expression due to the downstream effect manifested via the regulation mediated by miRNAs.

The dataset used in the analysis was composed of 148 families and 290 case-control samples. Samples were collected by the TSGeneSEE consortium collection and the dataset at our disposal was a mix of four different populations (Hungarian, Polish, Italian, Greek). In order to properly analyze the dataset, we had to separate the families from the case-control samples and check for population stratification. We analyzed the families using a TDT test while for the case-control genetic association we used a meta-analysis. The need to separate the two analyses resulted in a low-sample size that hindered the statistical power of the genetic study.

The Real-Time PCR experiments did not reveal any positive findings, while the OpenArray yielded some interesting results. The meta-analysis (Table 5) showed a significant association of rs3750486 ($p = 0.021$, MCPerm p-value = 0.001) located in the *LHX6* gene with TS. *LHX6* codes for a member of a large protein family expressed in the developing and adult mouse basal ganglia [321]. *LHX6* is thought to be a regulator of neuronal cells differentiation [322]. It is also a component of striatal activity modulation which is a brain region clearly linked to TS pathology [323, 324]. From a genetic point of view, *LHX6* has been already proposed as TS candidate gene [325]. We looked at linkage disequilibrium between the SNPs identified by the previous study [325] and the SNP detected in our 3'UTR analyses. We saw that they are not related ($D' = 0.293$; $r^2 = 0.004$). It is interesting to speculate, given the 3'UTR location of rs3750486, that we are detecting the genetic polymorphism that may alter the gene expression through a mechanism regulated by miRNAs. Indeed, rs3750486 of *LHX6* is in a putative target sequence of several miRNAs (mir-92a, mir-491, mir-5191), which might be disrupted by

the presence of the A allele, while the presence of the G allele might create a changed sequence possibly suitable for other miRNAs (mir-4447, mir-4472).

The second suggestive finding of our meta-analysis is rs7795011 (p = 0.029, MCPerm p = 0.009) at the *IMMP2L* gene. *IMMP2L* encodes for a protein that is involved in the disposal of signal peptide sequences needed to direct mitochondrial proteins in the mitochondria. There are a number of CNV studies linking *IMMP2L* to TS [20-23]. In this study, we found no genetic variations with a putative connection to miRNA regulation.

The TDT test analysis revealed a positive TS association with rs1042201 (p = 0.029) in the *AADAC* gene, the same gene picked up in the meta-analysis. *AADAC* is an enzyme involved in lipolysis and metabolism of drugs. It is principally expressed in the liver, the adrenal glands and the pancreas [326], therefore there is no clear indication about *AADAC* function in the human brain. A recent CNV genetic study, found positive association in a Danish TS cohort and the subsequent meta-analysis done over 1000 TS patients and 100,000 controls confirmed the role of *AADAC* deletions in TS pathogenesis [24]. Worth to mention, the genetic study was also reporting, at least in mice, the presence of *AADAC* protein in several regions of the human brain (hippocampus, corpus callosum and caudate nucleus) previously connected to Tourette Syndrome [327]. In addition, the 3'UTR SNP detected in our genetic study, rs1042201 is a putative target of mir-4263 which may play a role in human embryonic stem cells and neural forerunners [328].

SLITRK1, the most studied gene in Tourette Syndrome, did not yield a positive finding. The results of our genetic studies are consistent with many of the literature findings which investigated the role of *SLITRK1* in the etiology of TS and failed to detect brand-new variations as well as to duplicate the known ones [52-57]. It should be noted that the power of our study is limited by the small sample size, therefore is not surprising that we detected few positive associations. This phenomenon is usually referred to as the missing heritability, which states that most of the genetic variance is still elusive due to the small effect on the phenotype of interest. To fully uncover those effects, large sample sizes are needed. However, we observed a similar trend in the results of the TDT and the meta-analysis. So, it is possible to assume that our results are robust.

In conclusion, we identified positive association between rs1042201 in *AADAC* and significant association between rs3750486 in LHX6 and rs7795011 in *IMMP2L* genes with TS, thus providing further support for their possible biological role in the etiology

of TS. We are speculating that some of those influences may arise from miRNAs mediated modulation of gene expression. However, further association studies on larger populations as well as functional validation follow-up studies are warranted.

6.2 Epigenetic studies of Tourette Syndrome

In this project we wanted to investigate genome wide DNA methylation changes on blood samples of patients with a tic phenotype.

The samples were collected in the Netherlands and we described the first ever genome wide methylation analysis (EWAS) on tic disorders. One of the difficulties we first encountered was the establishment of an adequate sample size. The dataset was based on a self-reported questionnaire where patients would describe the manifestation of tics. The use of self-reported questionnaires is quite common. Indeed, in many cases, the medical centers responsible for the diagnosis and blood collection might be far from the hometown of the patients, therefore it is not possible to personally recruit or examine all the participants during the manifestation of tics [329], and clinicians rely on a self-report questionnaire to have an general overview of the symptoms.

The second factor we had to consider during the analysis of the dataset was represented by the heterogeneity: we had to correct for several factors like age, smoking, sex, tics, cell type and the position on the array. Indeed, it is known that all of these elements, with the exception of tics, affect DNA methylation levels [330-332].

Once the dataset was cleaned, we performed the EWAS. We did not find any significant CpG site after correcting for multiple tests. However, we were able to identify 57 CpGs that had an interesting p-value (<0.0001). The gene-ontology analysis and the subsequent literature research of the top CpGs, showed that many CpG sites are in proximity to genes previously associated with psychiatric or neurological disorders.

For instance, two CpGs (cg08093277 and cg21879791) are located near the gamma-aminobutyric acid type B receptor subunit 1 (*GABBR1*) gene which encodes for a component of the major inhibitory neurotransmitter (GABA) in the central nervous system and it has been associated to several neuropsychiatric disorders [333-336].

Moreover, we identified genes linked to Alzheimer's disease as BLM RecQ like helicase (*BLM*) and ADAM metallopeptidase domain 1A (pseudogne) (*ADAM1*) [337, 338] or schizophrenia as for clathrin interactor 1 (*CLINT1*) [339, 340]. More examples are SLC25A48 solute carrier family 25 member 48 (*SLC25A48*) which is a susceptibility gene for PD [341] and pleckstrin homology and RhoGEF domain containing (*PLEKHG3*) gene which is linked to mental retardation [342, 343]. Furthermore, some of the 57 CpGs point towards brain specific genes. For instance, syntrophin gamma 1 (*SNTG1*) connected to tic phenotype and that is expressed in the central nervous system [344] or Semaphorin-4G (*SEMAG4G*) which may be involved in cerebellar development [345].

Interestingly, two of the 57 CpGs (cg12961733 on *BRD1* and cg03573179 on *BRPF3*) lead to the involvement of *MOZ/MORF*, a histone acetyltransferase structure that modulates the dentate gyrus [346]. Acetylation is a post-translational modification that counteracts the positive charge of the histone tails amino group, thus resulting in the switch from a compacted chromatin structure (repression of transcription) to a more relaxed one (transcriptional activation). The MOZ/MORF subunit is made of bromodomain PHD finger protein (*BRPF1/2/3*), inhibitor of growth 5 (*ING5*) and homolog of Esa1-associated factor 6 (*hEAE6*) [347]. Moreover, bromodomain containing 1 (*BRD1*) which is linked to schizophrenia and bipolar disorder [206, 348], and bromodomain and PHD finger containing 3 (*BRPF3*) are components of the histone H3 acetyltransferase activity within the MOZ/MORF complex [349]. Furthermore, one CpG site is located near histone cluster 1 H4 family member e (*HIST1H4E*) gene, a member of histone 4 family which is a subunit of the nucleosome. The nucleosome modulated biological processes like transcription and DNA replication by compacting the DNA into chromatin.

The gene ontology analysis and the literature research have shown an involvement of epigenetic and neuropsychiatric genes. However, further studies on larger sample size or a more defined phenotype are warranted [121].

For example, future studies could consider a different approach that would include not only tics but also neurodevelopmental disorders that usually appears with TS such as OCD, ADHD and autism. Moreover, a longitudinal approach should also be taken into consideration to investigate at what stage of the disorders each environmental factor starts to affect the DNA methylation levels.

6.3 Abnormal Involuntary Movements (AIMs) animal model

In the transcriptome and methylome study of the AIMs animal model we wanted to investigate the region directly related to the manifestation of tics: the striatum.

The use of the animal models represents a very good option to perform molecular and biological studies in brain tissues involved in the pathogenesis of neurological disorders. Tourette Syndrome has a very complex and heterogeneous phenotype: the tics appears in situation of stress, the intensity and the severity of tics is always different, each patient manifest unique symptoms. It is impossible to have a single animal model capable of resembling all these facets. So, we decided to build the animal model based on the pathological situation observed in TS patients, a state of hypersensitivity to dopamine. The animal model was established by our collaborators at Boehringer Ingelheim. The behavioral data (Fig. 8) showed that administration of L-DOPA to the hypersensitive striatum triggers the appearance of so-called tics. It was originally planned as a Tourette Syndrome animal model but, after thinking about the biological background, observing the phenotype and discussing with experts in the field, we agreed that the model would not fit Tourette Syndrome but would fall under a different classification: abnormal involuntary movements (AIMs).

By using brain samples from this AIMs animal model, we wanted to investigate the effect of L-DOPA treatment on the locomotor activity as well as the counteracting effect mediated by the administration of Riluzole, a glutamatergic compound already implicated in the attenuation of AIMs. Indeed, the behavioral data showed that treatment with Riluzole reduced the locomotor activity (Fig. 8).

The transcriptome experiment showed that Riluzole treatment results in changes in the mRNA expression of those genes that are linked to the appearance of abnormal locomotor activity. The same genes which are highly expressed after the administration of L-DOPA. The mRNA data showed that Riluzole is able to counteract the effect mediated by L-DOPA on gene expression changes. Surprisingly, we observed that CREB1 was the top upstream regulator in all the groups investigated. Therefore, we decided to focus only on *CREB1* target genes in order to build a molecular network that might explain the effect of L-DOPA and Riluzole on gene expression (Fig. 10).

The molecular landscape indicates that the regulation of the apoptotic process has a pivotal role in both the appearance of AIMs after L-DOPA administration and in the

reduction of locomotor activity seen after Riluzole treatment. In general, the *CREB1* target genes lead to the hypothesis that L-DOPA administration leans towards increased neuronal survival, while the Riluzole treatment triggers apoptotic processes. Worth to mention, many of the genes depicted in the landscape encode for anti-apoptotic proteins. The same genes that are upregulated by L-DOPA administration were downregulated by the Riluzole treatment. Specifically, as shown by the upstream regulators (Table 10), the neuronal reaction to Riluzole is carried out via decreased activity of *CREB1*. So, *CREB1* acts as a remote controller with "go-between" commands. For instance, the activation of *CREB1* is modulated by phosphorylation mediated by the kinase ERK1/2 [350] and protein kinase C (PKC) [351]. The activation of *CREB1* results into the upregulation of anti-apoptotic genes and ultimately in less apoptotic process [352]. On top of that, 6-OHDA-lesioned rats followed by L-DOPA showed a surge in striatal neuron *CREB1* phosphorylation [353, 354]. To conclude, a recent study showed that *CREB1* phosphorylation is reduced by Riluzole [355].

The exact mechanism explaining how Riluzole would reduce the activity of *CREB1* is still unknown. By looking at what is reported in the literature and at the transcriptome data, we were able to propose a mechanism (Fig. 17) that might explain this mystery. In our opinion, Riluzole reduces the activity of *CREB1* leading to a modulation of the apoptotic process, and ultimately lower AIMs. To start with, Riluzole has many anti-glutamatergic properties [356-359] that negatively regulate the glutamatergic neurotransmission. The release of glutamate is able to trigger the post-synaptic NMDA receptors function, thus leading to the activation *ERK1/2*. The upregulation of *ERK1/2* leads to *CREB1* phosphorylation/activation [350]. To recapitulate, Riluzole would decrease the activity of *CREB1* by decreasing the *ERK1/2* signaling modulated by NMDA receptor. To continue, Riluzole is a strong inhibitor of *PKC* [360], a kinase directly involved in the phosphorylation/activation of *CREB1* [351]. Moreover, *PKC* activates *CREB1* also indirectly by positively modulating the NMDA receptors, thus leaning on the effect mediated by active ERK1/2 [361].

It is important to remember that even though the transcriptome data provided very interesting results, further studies to confirm this molecular mechanism are warranted. Indeed, we were not able to confirm that Riluzole leads to reduced *CREB1* activity by its phosphorylation. However, the data available in the literature support the hypothesis that

the phosphorylation of *CREB1* is a pivotal contributor in the manifestation of AIMs. For instance, several drugs such as amphetamines, cocaine, and the anti-psychotic aripiprazole (all involved in the appearance of AIMs) [362-364] trigger the activation of neuronal *CREB1* throughout a mechanism of phosphorylation [365-367]. To conclude, new studies could target *CREB1* activity/phosphorylation to investigate the modulation of AIMs.

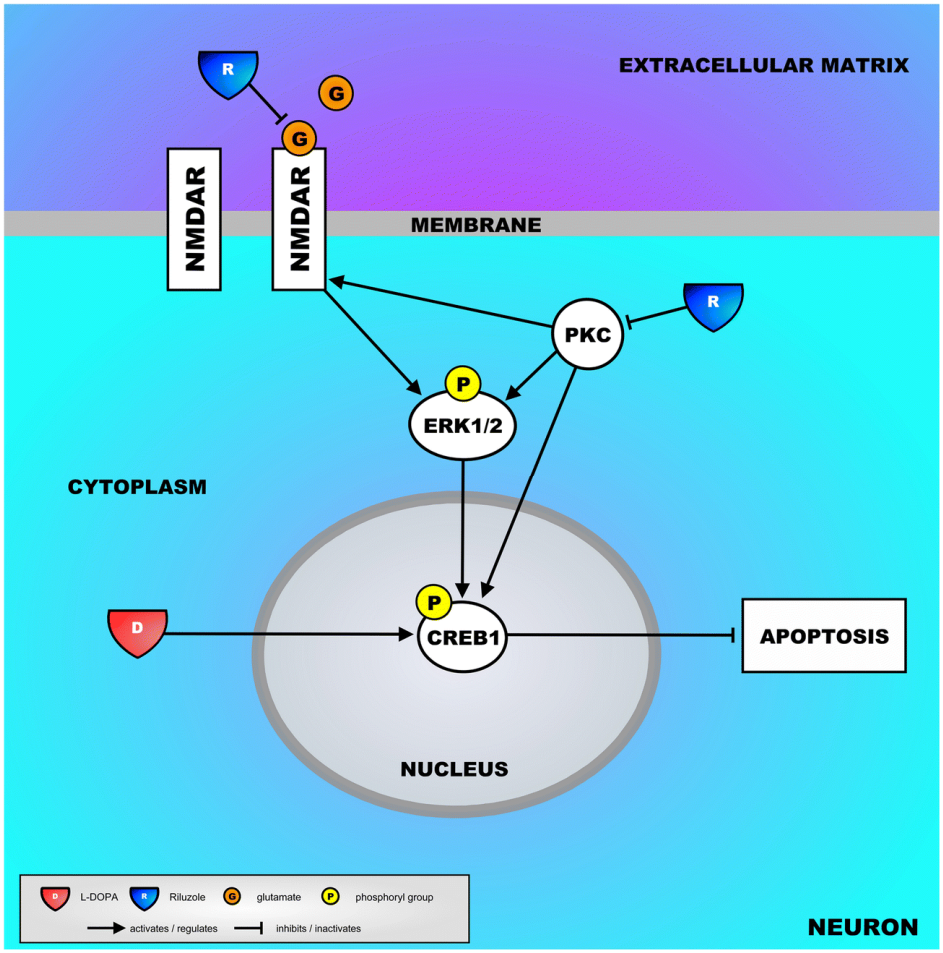

Fig. 17 from [151]

Schematic representation of the molecular mechanism of action of Riluzole via *CREB1* activity. The appearance of AIMs is affected by *CREB1* activity and its role in the modulation of the apoptotic process. Riluzole blocks the glutamate pathway leading to reduced *ERK1/2* signaling and diminished *CREB1* activity. Riluzole could also block *PKC* resulting in the reduction of *CREB1* activity.

In the DNA methylation experiments, we wanted to investigate genome wide DNA methylation pattern that might influence the appearance of locomotor activity. It is widely known that DNA methylation usually correlates to gene expression: hypomethylation leads to gene expression while hypermethylation leads to gene repression. The area nearby gene promoters are often enriched in stretches of DNA with high CpG density (CpG islands), which play a pivotal role in modulating gene expression changes. Considering the role of DNA methylation in transcriptional regulation we thought that abnormal methylation patterns would influence the appearance of locomotor activity observed after chronic treatment with L-DOPA. Supporting our hypothesis, a recent paper has shown how methylation regulates the appearance locomotor activity in a L-DOPA induced dyskinesia (LID) animal model [244].

We used a method called RRBS, which was not fully annotated on the rat genome, thus making the bioinformatics analysis more complicated given the absence of basic information on rodent methylation patterns. We observed an overall decrease of methylation (linked to higher gene expression) and discovered that most of the changes were located outside promoters/CpG islands.

Interestingly, the same genomic distribution observed in our study was also found in a paper investigating DNA methylation changed in the L-DOPA induce dyskinesia animal model [244]. Functionally speaking, those findings make sense with the pathological manifestation of locomotor activity. Indeed, the 6-OHDA injected rodents show a pretty normal behavior in absence of L-DOPA treatment which, once administered, leads to changes in gene expression and tic manifestation (as shown by the transcriptome data). In addition, intergenic areas of the genome are thought to be involved in the modulation of transcription levels and alternative splicing therefore it is possible to speculate that they might contribute to the abnormal transcriptional expression observed in animal model of AIMs/LID [244, 368-371]. Moreover, the extensive transcriptional changes observed in the mRNA data is unlikely explained only by changes in promoter region which are usually easily accessible by definition.

The DMSs analyses revealed that treatment (L-DOPA and Riluzole) administration has an influence on DNA methylation levels, mostly resulting in hypo-methylation which subsequently could lead to gene expression and might result in the manifestation of locomotor activity. Moreover, the comparison between lesioned and contralateral

striatum clearly demonstrated that 6-OHDA injection (lesioned side) has no significant effects on dynamic methylation changes. In addition, the data clearly showed that those changes in DNA methylation happen regardless the type of treatment: L-DOPA or L-DOPA + Riluzole. However, the comparison between the two different treatments (G1 vs. G3) did not show that many changes. It is interesting to speculate, based also on behavioral and mRNA data, that we are detecting the counteracting effect of Riluzole on gene expression which might be reflected also by less overall changes in DNA methylation detected when the two treatments were compared.

We also identified some regions with high density of DMSs and the following analysis focused only on high methylation changes (at least 80%) was able to identify few genes undergoing drastic variations in DNA methylation levels. It is worth to mention that we detected few genes because of the very stringent criteria used in the analysis as well as the difficulties in annotating the DMSs to a gene. Indeed, we were able to annotate only ~30% of the detected CpGs (Table 19). However, the biological relevance of the genes that we were able to identify was confirmed by a literature research that showed how many of them were already involved in brain disorders or functions: *Asic2* [372], *Itih4* [373], *Mad1l1* [374], *Mtm1* [375], *Robo1* [376, 377], *Disc1* [378-380], *Hip1* [381].

To gain some more knowledge about the overall biological function of all the CpGs detected by RRBS, we performed gene ontology analysis which indicated an enrichment in genes involved either in psychiatric disorders, glutamate neurotransmitter system or brain functions.

For example, we detected Netrin 4 (*NTN4*), which is a gene involved in the formation of neurites that has been already implicated in the pathogenesis of TS in two genetic association studies [49, 325]. Another gene already associated with TS [27, 32] is Neurexin 1 (*NRXN1*), which encodes for a pre-synaptic cell-adhesion molecules involved in the growth of synapses and in the transmission of glutamate and GABA signals.

An interesting gene is phosphodiesterase 10A (*PDE10A*), which belongs to a family involved in the regulation of cyclic nucleotide (*cAMP*) and that might influence locomotor movements as well as the expression of *CREB* [137, 382]. Another example is Reelin (*RELN*), which modulates neuronal microtubules as well as the migration of neurons and it has been associated with autism [383] and schizophrenia [384].

We also identified two genes (*GRM4* and *GRM8*) belonging to a metabotropic glutamate receptor family and two genes (*GRIK3* and *GRIK4*) involved in the modulation of glutamate, the major excitatory neurotransmitter in the central nervous system. In particular, *GRM4* and *GRM8* are implicated in the inhibition of the cyclic AMP cascade and they have been linked to epilepsy, depression, schizophrenia, depression, and alcohol abuse [385-388]. On the other hand, *GRIK3* and *GRIK4* function as ligand-activated ion channels and have been linked to depression and alcohol abuse [389, 390].

The involvement of glutamate was corroborated by a family of genes (*CACGN4* and *CACGN8*) related to glutamate neurotransmission, by the regulation of AMPA receptor activity, already involved in schizophrenia [391]. One direct link to epigenetics is represented by *DNMT3A*, a gene that encodes for a DNA methyltransferase involved in the establishment of new methylation pattern. It is possible to speculate that abnormalities in the function of DNA methyltransferase might lead to prolonged changes in gene expression behind the manifestation of involuntary locomotor activity.

Furthermore, we identified Serum/glucocorticoid regulated kinase 1 (*SGK1*), a serine/threonine kinase activated by the PI3K signal which is highly expressed in the neurons of several diseases such as PD, Huntington's disease and amyotrophic lateral sclerosis [392-395]. We also detected methylation changes in 3 CpGs nearby Cadherin 13 (*CDH13*), which is involved in the negative regulation of axon growth and it may also protect endothelial cells from oxidative stress induced apoptosis it has been found hyper-methylated in many types of cancer [393] and linked to five of the major neuropsychiatric disorders: autism, schizophrenia, bipolar disorder, depression and ADHD [396]. Interestingly, glutamate is a frequent term in the gene enrichment analysis. It is quite normal to find glutamate in the group treated with Riluzole since it is a glutamatergic drug, but it is very surprising to find glutamate also in the group treated only with L-DOPA. The result of our analysis provides further support to the hypothesis of glutamate in the development of TS already suggested by both genetic and neuroimaging studies [70].

A similar enrichment has been identified by previous studies on dynamic DNA methylation [244, 368, 369, 397]. In our case, it is very interesting to note that treatment with L-DOPA only resulted in a high enrichment of glutamate terms, thus supporting the theory of glutamate in the pathogenesis of Tourette [70].

We also checked the genes identified in the methylation studies with the mRNA expression data and we did not find a direct and significant correlation between gene expression and methylation levels. This is consistent with other studies showing that some brain areas show no correlation between DNA methylation and mRNA expression [398-400]. In our case, the genes for which we had data were either not picked up or not significantly expressed in the mRNA data.

The identification of a single CpG in the proximity of a gene is usually not sufficient to correlate DNA methylation changes with the manifestation of a certain phenotype (locomotor activity in our case). It is important to remember that we are looking only at each single CpG in the exact moment when the samples were collected, the same CpG is then annotated to a nearby gene and we assume that any eventual variation of methylation will result in gene expression changes. However, in most of the cases, these assumptions do not necessarily represent the actual biological situation even though they constitute an important indication of the methylation profile. It is the main reason that usually brings researchers to perform some independent validation to strengthen the results. We could not perform any validation experiments, but we are very confident that the CpGs identified in the study reflect real changes since we used an extremely stringent threshold. By adopting such stringent parameters, we opted for specificity over selectivity and decided to be able to detect only real changes even though it would result in the potential loss of many CpGs. The main reason of such stringent threshold is the complexity of the analysis and the need to identify changes due to 6-OHDA injection, L-DOPA administration, Riluzole treatment. Finally, it important to remember that the DNA methylation data might suffer from limitation by sample size since we were able to analyze only 3 rodents for each experimental group and more experiment, as well as independent single gene validation studies, are definitely needed to increase the knowledge on the effect of 6-OHDA, L-DOPA and Riluzole on DNA methylation changes. However, considering the stringent criteria used in the analysis, the findings so far discussed are strong and they might be used for further validation studies.

To conclude, we were able to show that chronic drug treatment can induce DNA methylation changes. It might be one of the causes involved in the appearance and intensity of abnormal locomotor activity. These dynamic changes are not influenced by the side of the brain (lesioned or contralateral).

7. CONCLUSION

7.1 Genetic studies of Tourette Syndrome

In this project, we looked at genetic variation in 3'UTR of TS candidate genes with a putative connection to binding sites for miRNA. The Real-Time PCR did not find any positive results. The meta-analysis detected a positive association between rs3750486 ($p = 0.021$) located on *LHX6* and rs7795011 ($p = 0.029$) located on *IMMP2L* with TS. Moreover, the TDT test also picked up an over-transmission of the A allele of rs1042201 ($p = 0.029$, $\chi 2 = 4.787$) located on *AADAC* and TS patients. These finding confirm the literature evidence already proposing these genes as some of the many components in the TS pathology. The genetic association study also showed how lower sample size and population stratification hamper statistical power and it emphasized the need of a large collaborative effort in order to overcome those limitations.

Worth to mention, to solve the genetic turmoil, more studies on different population are needed since it is likely that many of the genetic variations propelling the development of TS might differ based also on the geographic area, as observed in many studies. This population difference might also help to explain the lack of replicating findings between different studies.

In addition, to prove the actual role of miRNAs in the abnormal regulation of those genes as consequence of genetic variation (e.g. SNPs) functional validation studies are warranted.

7.2 Epigenetic studies of Tourette Syndrome

In this project, we looked at methylation differences on a genome-wide levels to study and identify some CpGs that might be involved in the manifestation of tics. The EWAS analysis did not find any significant methylation changes even though it provided some interesting genes for follow-up studies. However, it would be optimal to increase the sample size (to have more statistical power) or to better define the phenotype of TS patients (to have more defined analyses). To conclude, we showed that epigenetic may be one of the many factors contributing to the development of Tourette Syndrome.

7.3 Abnormal Involuntary Movements (AIMs) animal model

In this project, we looked at the effect of L-DOPA administration and Riluzole treatment on mRNA and DNA methylation in an animal model of AIMs. The behavioral data showed that Riluzole reduced the appearance of involuntary movements. The mRNA data demonstrated that Riluzole completely overturns the expression of genes involved in the regulation of apoptosis. The molecular landscape identified *CREB1* (activation through phosphorylation) as the pivotal player in the appearance/reduction of AIMs. However, this molecular mechanism requires follow-up studies to be confirmed. It could be considered to test new/existing treatment for AIMs.

On the other side, the DNA methylation studies did not provide converging results as the transcriptome analysis. However, we gained knowledge on the changes in DNA methylation levels in animal model of AIMs. Indeed, the DMSs analysis revealed that lesioned and contralateral striatum have almost identical methylation profiles. In addition, we clearly observed that various treatments (L-DOPA administration or Riluzole treatment) had no differential effect on the methylation profile (lesioned or contralateral). Furthermore, the gene ontology showed the likely contribution of glutamate in the appearance of locomotor activity and it also provided different genes for follow-up studies.

8. SUMMARY

Tourette Syndrome is a neurodevelopmental disorder characterized by the appearance of involuntary locomotor and vocal activity (tic). The etiology of TS is still far from being understood. The European union decided to approach this disorder with a team made of 12 PhD students spread in universities and pharma companies (TS-EUROTRAIN).

The PhD dissertation described above originates from the extensive exchange of individual skills, scientific knowledge and biological samples.

In this PhD project we screened genetic variations in the 3'UTR of TS candidate genes. The use of meta-analysis and TDT test, as required by the dataset at our disposal, detected three TS positive association. In details, we picked up rs3750486 located in LHX6, rs7795011 located in *IMMP2L*, and rs1042201 located in *AADAC*. These genetic findings confirm the literature evidences linking the genes with TS pathology.

We also looked, for the first time, at genome wide methylation differences in blood samples of patients manifesting tic phenotype. The analysis did not find any significant CpG site even though it provided some interesting genes for follow-up studies. For examples: *GABBR1* gene linked to the major inhibitory neurotransmitter (GABA) in the central nervous and the MOZ/MORF complex.

In addition, we investigated effect of L-DOPA administration and Riluzole treatment on mRNA and DNA methylation profile of Abnormal Involuntary Movements (AIMs) rodent model. The behavioral data clearly showed that Riluzole is able to stop the appearance of locomotor activity. The molecular landscape built using mRNA data identified *CREB1* (its activation through phosphorylation) as a pivotal player in the appearance/reduction of AIMs.

On the other side, the DNA methylation studies did not provide the same converging results as the transcriptome analysis. However, we gained knowledge on the changes in DNA methylation levels in animal model of AIMs. Indeed, the DMSs analysis revealed that lesioned and contralateral striatum have almost identical methylation profiles. In addition, we clearly observed that various treatments (L-DOPA administration or Riluzole treatment) had no differential effect on the methylation profile (lesioned or contralateral). Furthermore, the gene ontology showed the likely contribution of glutamate in the appearance of locomotor activity and it also provided different genes for follow-up studies.

9. ÖSSZEFOGLALÁS

A Tourette-szindróma (TS) akaratlan mozgásszervi és hangbeli aktivitással (ún. tic) járó idegfejlődési rendellenesség. A TS etiológiája még nem teljesen feltárt. Az Európai Unió megbízásából egy TS-EUROTRAIN elnevezésű, több egyetemről és gyógyszercégtől jövő 12 PhD hallgatóból álló csoport foglalkozik a rendellenesség megértésével. A fent tárgyalt PhD disszertáció az egyéni képességek, tudományos tudás és biológiai minták széleskörű, kölcsönös cseréjéből származik. Jelen PhD projekt során TS-kandidáns gének 3' UTR részében található genetikai variációk vizsgálatával foglalkoztunk. A rendelkezésre álló adathalmazon végzett meta-analízis és TDT-teszt 3 TS-pozitív asszociációt mutatott ki: az *LHX6*-ban elhelyezkedő rs3750486, az *IMMP2L*-ben elhelyezkedő rs7795011és az *AADAC*-ben elhelyezkedő rs1042201. Ezek a genetikai találatok megerősítik az irodalmi adatokat, melyek szerint ezek a gének kapcsolatba hozhatók a TD patológiájával. Elsőként mi vizsgáltuk meg a genomszintű metilációs különbségeket tic-fenotípussal rendelkező betegek vérmintáiban. Az analízis nem tárt fel CpG-helyekhez kapcsolódó szignifikáns különbségeket, de a találatok között van néhány érdekes gén, melyeket érdemes tovább követni, például a *GABBR1* gén, mely a központi idegrendszer fő gátló neurotranszmitteréhez (GABA) kapcsolható és a MOZ/MORF komplex. Továbbá, megvizsgáltuk az L-Dopa- és riluzol-kezelés hatását mRNS és DNS-metilációs szinten AIM („abnormális akaratlan mozgások") rágcsáló modellben. A viselkedési tesztek adatai egyértelműen megmutatták, hogy riluzol képes megállítani a mozgásszervi aktivitás megjelenését. Az mRNS adatokból megalkotott molekuláris tájkép a *CREB1*-et azonosította, mely – foszforilációval történő aktivációja által – kulcsszerepet játszik az AIM megjelenésében és eltűnésében. Másrészről, a DNS-metilációs vizsgálatok nem mutattak olyan összefüggő eredményeket, mint a transzkripciós analízis. Azonban tudást szereztünk a DNS-metilációs szintek változásáról AIM egérmodellben. A DMS-analízis feltárta, hogy a léziós és az ellentétes oldali striatum metilációs profilja közel azonos. Továbbá, egyértelműen kimutattuk, hogy a különböző kezeléseknek (L-Dopa vagy riluzol) nincs megkülönböztető hatása a metilációs profilra nézve (léziós és ellenoldali). Végül, a génontológia a glutamátot egy lehetséges közreműködőként azonosította a mozgásszervi aktivitás megjelenésében, és különböző géneket tárt fel további analízisekhez.

10. PHD CANDIDATE'S PUBLICATIONS

10.1 Publications discussed in the PhD dissertation

1) Riluzole Attenuates L-DOPA-Induced Abnormal Involuntary Movements Through Decreasing CREB1 Activity: Insights from a Rat Model. **Pagliaroli L**, Widomska J, Nespoli E, Hildebrandt T, Barta C, Glennon J, Hengerer B, Poelmans G. Mol Neurobiol. 2018 Nov 27. doi: 10.1007/s12035-018-1433-x. [Epub ahead of print] PubMed PMID: 30484112. **IF 5.026**

2) From Genetics to Epigenetics: New Perspectives in Tourette Syndrome Research. **Pagliaroli L**, Vető B, Arányi T, Barta C. Front Neurosci. 2016 Jul 12;10:277. doi: 10.3389/fnins.2016.00277. eCollection 2016. Review. PubMed PMID: 27462201; PubMed Central PMCID: PMC4940402. **IF 3.556**

3) Epigenome-Wide Association Study of Tic Disorders. Zilhão NR, Padmanabhuni SS, **Pagliaroli L**, Barta C; BIOS Consortium, Smit DJ, Cath D, Nivard MG, Baselmans BM, van Dongen J, Paschou P, Boomsma DI. Twin Res Hum Genet. 2015 Dec;18(6):699-709. doi: 10.1017/thg.2015.72. Epub 2015 Oct 26. PubMed PMID: 26499864. **IF 2.339**

4) TS-EUROTRAIN: A European-Wide Investigation and Training Network on the Etiology and Pathophysiology of Gilles de la Tourette Syndrome. de la Tourette Syndrome. Forde NJ, Kanaan AS, Widomska J, Padmanabhuni SS, Nespoli E, Alexander J, Rodriguez Arranz JI, Fan S, Houssari R, Nawaz MS, Rizzo F, **Pagliaroli L**, Zilhäo NR, Aranyi T, Barta C, Boeckers TM, Boomsma DI, Buisman WR, Buitelaar JK, Cath D, Dietrich A, Driessen N, Drineas P, Dunlap M, Gerasch S, Glennon J, Hengerer B, van den Heuvel OA, Jespersgaard C, Möller HE, Müller-Vahl KR, Openneer TJ, Poelmans G, Pouwels PJ, Scharf JM, Stefansson H, Tümer Z, Veltman DJ, van der Werf YD, Hoekstra PJ, Ludolph A, Paschou P. Front Neurosci. 2016 Aug 23;10:384. doi: 10.3389/fnins.2016.00384. eCollection 2016. PubMed PMID: 27601976; PubMed Central PMCID: PMC4994475. **IF IF 3.556**

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10.2 Publications not discussed in the PhD dissertation

1) Reduced expression of MYC increases longevity and enhances healthspan. Hofmann JW, Zhao X, De Cecco M, Peterson AL, **Pagliaroli L**, Manivannan J, Hubbard GB, Ikeno Y, Zhang Y, Feng B, Li X, Serre T, Qi W, Van Remmen H, Miller RA, Bath KG, de Cabo R, Xu H, Neretti N, Sedivy JM. Cell. 2015 Jan 29;160(3):477-488. doi: 10.1016/j.cell.2014.12.016. Epub 2015 Jan 22. PubMed PMID: 25619689; PubMed Central PMCID: PMC4624921. **IF 28.710**

2) Summaries of plenary and selected symposia sessions at the XXIV World Congress of Psychiatric Genetics; Jerusalem, Israel; 30 October 2016-3 November. Ciobanu LG, Ori AP, **Pagliaroli L**, Polimanti R, Spindola LM, Vincent JB, Cormack FK. 2016. Psychiatr Genet. 2017 Apr;27(2):41-53. doi: 10.1097/YPG.0000000000000167. PubMed PMID: 28212207. **IF 1.586**

3) Summaries of plenary, symposia, and oral sessions at the XXII World Congress of Psychiatric Genetics, Copenhagen, Denmark, 12-16 October 2014. Aas M, Blokland GA, Chawner SJ, Choi SW, Estrada J, Forsingdal A, Friedrich

M, Ganesham S, Hall L, Haslinger D, Huckins L, Loken E, Malan-Müller S, Martin J, Misiewicz Z, **Pagliaroli L**, Pardiñas AF, Pisanu C, Quadri G, Santoro ML, Shaw AD, Ranlund S, Song J, Tesli M, Tropeano M, van der Voet M, Wolfe K, Cormack FK, DeLisi L. Psychiatr Genet. 2016 Feb;26(1):1-47. doi: 10.1097/YPG.0000000000000112. PubMed PMID: 26565519. **IF 1.557**

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I started this PhD project in April 2014.
Since then, I had the opportunity to do internships in Germany (Boehringer Ingelheim), Iceland (deCODE Genetics), Netherlands (Drug Target ID). I have been attending workshops and conferences. I have been playing basketball for Semmelweis University and for Accenture (Uzleti liga). I worked in a vibrant and beautiful city.

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I also spent 439 days traveling in South East Asia. I taught English to Vietnamese students, I worked in a farm in Australia, I have been a receptionist for a hostel in Bangkok. I drove around South East Asia. I swam in crystal clear waters. I picked up trash in Thailand. I witnessed the beauty of simplicity. I have been charmed by Myanmar. I met incredible people.

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