Abstract

Background

Genetic research in neurodegenerative disorders identified candidate genes in many familial cases, providing insight into the pathogenesis. Many of them share common pathologic mechanisms which may result from alterations in gene expression. Single gene disorders can be great models for complex neurodegenerative pathophysiology, however, questions remain, and progress in their treatment is still a challenge.

This research project started in the clinic, with a family with an apparently genetic neurodegenerative disorder, including hemiplegic migraines and systemic serotonin deficiency.

Serotonin has roles in physiological and behavioural functions, neurodevelopment, as well as neuropsychiatric, gastrointestinal and cardiovascular disorders and migraines. Serotonin metabolism includes synthesis, transport and degradation.

Methods

Building hypothesis: started with translational research, taking basic science into practical applications. To answer the question of the systemic serotonin deficiency, genes of serotonin synthesis, transport and developmental pathway were sequenced, and platelet serotonin uptake studies were performed. Furthermore, SERT protein, actin and other cytoskeleton proteins were quantified with Western blots and proteomic analysis, in different platelet fractions.

Generating hypothesis: continued with Whole Exome Sequencing. For validation, transcriptome analysis was performed.

Along with investigations, treatment with serotonin replacement, 5-hydroxytryptophan was started in the patients.

Results

Sequencing was negative for genes associated with serotonin production, transport, and development. Serotonin uptake was deficient in patient platelets. Western blots showed diminished cytoskeleton proteins in Triton-soluble platelet fractions.

WES analysis yielded a candidate gene, SRRM2, a splicing coactivator protein with potentially damaging missense mutation. Transcriptome analysis revealed several differentially expressed genes.

Treatment with serotonin replacement significantly improved the lower limb strength.
Conclusions

Cytoskeleton proteins were aggregated trapping membrane proteins inside the cell, explaining the biochemical phenotype. The transcriptome analysis demonstrated significant changes in transcription levels in multiple ontology pathways. Some of these genes are known to cause neurodegenerative diseases.

The changes in the transcription levels suggest that a defect at the core of the spliceosome complex is a reasonable hypothesis explaining the basis of this family’s neurodegeneration.

The success with serotonin treatment is also new and deserves further research.
Preface

Choosing Experimental Medicine at UBC as my graduate program seemed to be the perfect fit for a research incorporating equally clinical and laboratory work. This choice was done together with my supervisory committee.

All laboratory experiments presented in this thesis were designed, performed and interpreted by me, with the supervision of my advisors. A few of the Western blots were performed by the Nashville Lab, but later repeated by myself, and this is stated clearly in the thesis. The MRI’s and pathology slides were performed on clinical basis. The transcriptome analysis was interpreted also by me, based on information from various available webinars.

The clinical case description and platelet serotonin uptake studies were published in the journal Cephalalgia (G Horvath, K Selby, K Poskitt, K Hyland, P Waters, M Coulter-Mackie, S Stockler-Ipsiroglu, Hemiplegic migraine, seizures, progressive spastic paraparesis, mood disorder, and coma in siblings with low systemic serotonin. Cephalalgia 31(15) 1580–1586). I wrote the case report and I did perform the platelet serotonin uptake analysis. I also made the graph with the serotonin uptake that has been published and appears in Chapter 3 (Figure 21). Dr. Selby contributed with clinical care, Dr. Poskitt reviewed the MRI’s, Dr. Hyland performed the CSF neurotransmitter analysis, and Dr. Coulter-Mackie, Waters and Stockler-Ipsiroglu are my supervisors. They supervised the experiments and helped with editing the manuscript. All authors read and approved the published paper.

There are two manuscripts in preparation, the first is incorporating the results of the cytoskeleton experiments and transcriptome analysis, and it is written in collaboration with the Blakely Lab; the second is presenting the anatomical pathology findings on the autopsy, written in collaboration with Dr. Wayne Moore, Neuropathologist at Vancouver General Hospital and Dr. Peter Schutz, senior Pathology Resident.

The results of this research have been presented at the Clinician Investigator and Experimental Medicine Programs Student Research days, and as oral presentations at several National and International meetings and conferences (at the 12th International Congress of Inborn Errors of Metabolism in 2013, at a Movement Disorders in Childhood meeting in Rome in 2013, and at the Canadian College of Medical Geneticists 39th Annual Scientific Conference in 2014). There were several poster presentations at the Child and Family Research Institute Student Research days.
All experiments were conducted in the Marion Coulter-Mackie and Blakely Labs, with the University of British Columbia Ethics Board’s approval (Certificate # H07-02288).
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<th>Description</th>
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<tbody>
<tr>
<td>3OMD</td>
<td>3-O-methyldopa</td>
</tr>
<tr>
<td>5HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>5HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>5HTP</td>
<td>5-hydroxytryptophan</td>
</tr>
<tr>
<td>AADC</td>
<td>Aromatic L-amino acid decarboxylase</td>
</tr>
<tr>
<td>ABP</td>
<td>Actin binding proteins</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADF</td>
<td>Actin depolarizing factor</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BH2</td>
<td>Dihydrobiopterin</td>
</tr>
<tr>
<td>BH4</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CADASIL</td>
<td>Cerebral arteriopathy with subcortical infarcts and leukoencephalopathy</td>
</tr>
<tr>
<td>c-AMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CDB</td>
<td>Corticobasal degeneration</td>
</tr>
<tr>
<td>CMT</td>
<td>Charcot-Marie-Tooth</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CS</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>CSD</td>
<td>Cortical spreading depression</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropteridine reductase</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>ESE</td>
<td>Exonic splicing enhancer</td>
</tr>
<tr>
<td>Fgf8</td>
<td>Fibroblast growth factor 8</td>
</tr>
<tr>
<td>FHM</td>
<td>Familial hemiplegic migraine</td>
</tr>
<tr>
<td>FTLD</td>
<td>Frontotemporal lobar dementia</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma amino-butyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPCH</td>
<td>Guanosine triphosphate cyclohydrolase</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>HDR</td>
<td>Hypoparathyroidism deafness renal anomalies</td>
</tr>
<tr>
<td>HSP</td>
<td>Hereditary Spastic Paraplegia</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanillic acid</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate filament</td>
</tr>
</tbody>
</table>
IP2

Phosphatidyl inositol 3

KEGG

Kyoto Encyclopedia of Genes and Genomes

Km

Michaelis constant

MAO

Monoamine oxidase

MAP

Microtubule associated protein

MELAS

Mitochondrial Encephalopathy Lactic acidosis and Stroke-like episodes

MND

Motor neuron disease

MOCA

Montreal Cognitive Assessment

MRI

Magnetic Resonance Imaging

mRNA

Messenger RNA

MRS

Magnetic Resonance Spectroscopy

MS

Multiple Sclerosis

NAD

Nicotinamide adenosine dinucleotide

ND

Neurodegenerative disorders

NMDA

N-methyl-d-aspartate

NO

Nitric oxide

NT

Neurotransmitter

ORF

Open reading frame

PD

Parkinson’s disease

PKB

Protein kinase B

PKC

Protein kinase C

pre-mRNA

Precursor messenger RNA

PSP

Progressive supranuclear palsy

PTPS

6-pyruvoyltetrahydrobiopterin synthase

RNA

Ribonucleic acid

RRM

RNA recognition domain

SBP

Serotonin binding protein

SCA

Spinocerebellar ataxias

SERT

Serotonin transporter

Shh

Sonic hedgehog

SIDS

Sudden infant death syndrome

SLC6

Solute carrier family 6

SMA

Spinal Muscular Dystrophy

snRNP

Small nuclear ribonucleoprotein

SRRM2

Serine arginine repetitive matrix 2

TPH

Tryptophan hydroxylase

TS

Triton soluble

UMN

Upper motor neuron

VMAT

Vesicular monoamine transporter

Vmax

Maximum velocity

WB

Western blot

WES

Whole exome sequencing

WHO

World Health Organization
Acknowledgements

With my thesis approaching its finalization, it was inevitable to reflect on the past and realize that along the long road that led me to this milestone I was not alone, I stayed on course guided and encouraged by many of my colleagues, friends and family.

First and foremost I am grateful to my supervisors that they have supported me, guided me, and encouraged me over these years, even through less successful periods. I would like to thank Dr. Marion Coulter-Mackie for her patience, wisdom, and understanding, for the endless teaching hours that she spent with me in her lab, for the sign “No gremlins allowed” as many experiments needed to be repeated, and for never giving up on me and my project.

I would like to thank Dr. Sylvia Stockler-Ipsiroglu, without her I would have never started this project, she is the reason that I took the clinical information from the bedside to the laboratory, expanded it into translational research and then taking it back to the patient. Her previous experience in clinical research was my inspiration all along.

The guidance and sobering wisdom of Dr. Paula Waters took me back to reality whenever my imagination wondered beyond possibilities of biochemistry and pathophysiology. I thank for her structured approach to designing difficult experiments that taught me the most needed discipline in working in a research laboratory, and I will always remember that.

I would like to offer my endless gratitude to the Blakely Lab in Nashville, Dr. Randy Blakely, Dr. Ran Ye, and Chris Switek. I cannot express how much it meant for me their kind acceptance of a clinician to such a world-renowned laboratory, taking me step by step through the various techniques and experiments. I consider those 12 weeks spent in the Blakely Lab one of the most wonderful experiences in my career.

I would like to thank Jennifer Lian in the Coulter-Mackie Lab for her patience and vast knowledge of all the ins- and outs of gel apparatuses, PCR settings, pH meters and scintillators. It was good to know that if I have a question she’s always there to answer.

I would like to thank the UBC Clinician Investigator Program and to the Rare Diseases Foundation for supporting this research.

I thank my family for accepting the long working hours, the busy weekends and time spent away, as a fact of life, and not a matter of complaints. To my husband for all the constructive criticism, to my boys for just being there, and to my daughter, who was my greatest teacher in formatting my thesis, the most difficult task after all. I love you all very much.
I would like to thank my friend Saadet Mahmutoglu for the encouragements during times when I didn’t think I would get through the day, but who was there to cheer when I had successes too.

My greatest admiration goes to the patients and the family, for their faith in me, their understanding of the risks and uncertainties that come with research. Their lives, and struggles, their attitude toward the quest for an answer fueled my unstoppable desire to look for an explanation of the unexplained. I learnt from them the most.
Dedication

To Grace
Chapter 1: Background

This research study started as a clinical research, at the bedside, with a family with a unique, but devastating neurodegenerative course involving three generations. Multiple family members were affected in similar way, but with different clinical severities. The common clinical features were: hemiplegic migraines of different severity, progressive spastic paraparesis, myoclonic seizures, sensory loss, dysarthria, autonomic dysfunction, and in some cases tumour development. In most cases there was no cognitive disability involved. Common MRI findings were T2 hyperintensities in the brain stem and a severely atrophied spinal cord, that was stable over time and most likely was developmental in origin. The proband ultimately had a severe episode of hemiplegic migraine, leading to coma that lasted for two months and left the patient with serious neurological sequelae with intractable seizures, and extensive white and grey matter injury on brain MRI, most likely caused by hypoxic ischemic damage that occurred during the coma. She died 15 months after the coma episode. Various biochemical investigations led to the common finding of severe systemic serotonin deficiency, with low levels in CSF and platelets. This prompted initiation of serotonin replacement therapy with 5-hydroxytryptophan, parallel to continuing further etiological investigations. The response to treatment was surprising and significant, with marked improvement in the lower limb strength, and return of ambulation. A hypothesis was formulated, looking at all the potential causes of systemic serotonin deficiency, starting from deficient synthesis (tryptophan hydroxylase enzyme deficiency), deficient transport (serotonin transporter defect), and storage (vesicular monoamine transporter defect), or a defect in the serotonin developmental pathway. Several candidate genes have been sequenced, and the serotonin transporter function was assessed with a platelet serotonin uptake assay. The transporter activity was low but there were no mutations found in the serotonin transporter gene. Further investigations on the transporter have found that it is trapped inside the platelets in cytoskeleton aggregates. Further hypothesis was that the cytoskeleton and scaffolding proteins should be involved, but with the advancement in methods of Next generation sequencing, we have started a hypothesis generating research, with Whole Exome Sequencing. The results were new, involving a gene at the centre of the spliceosome component, and for further characterizing this gene, transcriptome analysis was performed. This
resulted in significant findings that are important not only for this family, but have potentially brought light on the pathomechanism of other neurodegenerative disorders.

The surviving affected family members are still on treatment with serotonin replacement and although they have had no serious episodes of migraine, associated with coma, their clinical course is slowly deteriorating. Hopefully with more research and understanding fully the underlying mechanism of the neurodegeneration, we will be able to offer them a treatment that would eventually halt the disease process.

1.1 Neurodegeneration

Neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS), Parkinson’s disease (PD), Frontotemporal lobar dementia (FTLD), Alzheimer’s disease, Spinal muscular atrophy (SMA), Lewy body disease, and Huntington’s disease (HD) have many similarities and differences. Collectively they are a serious burden to health care in industrialized countries. The economic cost is enormous, reaching multiple trillion dollars per year according to WHO statistics (http://www.who.int/healthinfo/global_burden_disease/en/). Their insidious presentation and progressive nature make them hard to diagnose early and there have been no successful therapies found, other than symptomatic management and support into palliative care. Genetic research has identified several causative and candidate genes in familial cases of many of the neurodegenerative disorders, and over the last decade identification of mutations in these genes has provided insight into the pathogenesis of these diseases. However although efforts have been made to make individual genetic diagnoses, there are considerable number of neurodegenerative diseases without an associated single gene discovered and therefore multigenetic, environmental and behavioural factors are taken in consideration in their etiology.

ALS is a progressive neurodegenerative disease, presenting with lower limb weakness, and eventually paralysis, caused by degeneration of the motor cortex, corticospinal tracts, brainstem and spinal cord. Its onset is in adulthood and usually leads to death due to paralysis of respiratory muscles, causing respiratory failure in about 2-5 years 1.

SMA causes progressive weakness through loss of the motor neurons in the anterior horn of the spinal cord, resulting in muscle denervation, skeletal muscle atrophy and paralysis, especially of the proximal muscles. There are late onset milder forms, but most cases present in infancy 2,3.
Multiple Sclerosis (MS) is a progressive inflammatory demyelinating disorder, mostly presenting in adults. It is a heterogeneous disease in respect to clinical course and neuroradiological presentation. MS can present with lower limb spasticity and sensory disturbances, it can cause ataxia and bladder sphincter instability. There are white matter changes on brain imaging and there is frequent optic nerve involvement⁴.

Hereditary Spastic Paraplegia (HSP) syndromes are characterized by progressive lower limb spasticity and weakness. There are more than 50 genetic types described. It can present in late childhood or adulthood, sometimes associated with ataxia, dysarthria, or urinary urgency, and the progression rate is variable⁵.

Spinocerebellar ataxias (SCA) are multigenetic neurodegenerative disorders characterized by cerebellar and spinal involvement. Clinical features include ataxia, dysarthria, spasticity, optic atrophy, retinopathy, and extrapyramidal movement disorder⁶.

Parkinson’s disease (PD) is a common neurodegenerative disorder, causing bradykinesia, rigidity and tremor. Prior to the motor manifestations there are several non-motor, prodromal symptoms that include constipation, sleep disturbances, depression and autonomic dysfunction. The brain pathology shows an ascending involvement from the medulla to the neocortical areas, and the hallmark of the disease is dopaminergic neuronal destruction in substantia nigra and widespread accumulation of Lewy bodies⁷. There is also evidence of noradrenergic neuronal loss in locus coeruleus and corresponding sympathetic function loss⁸. Several studies in the last decade have shown also serotonergic neuronal loss that has been implicated in the development of motor and non-motor symptoms of Parkinson’s disease⁹.

Frontotemporal dementia (FTD), another neurodegenerative disorder, that has three main types, the behavioural, the progressive non-fluent aphasia, and semantic dementia types, has also been associated with motor disease. It has been also shown to cause neuronal cytoplasmic aggregation in the brain¹⁰.

Mitochondrial disorders are multiorgan diseases, may present with migraine, cerebellar signs and white matter signal abnormalities on MRI. They can be inherited through the mitochondrial DNA from the maternal lineage, or through autosomal recessive or dominant inheritance model. The mitochondrial disorder with stroke-like episodes mimicking hemiplegic migraine would be MELAS syndrome (Mitochondrial Encephalomyopathy Lactic acidosis and Stroke-like episodes).
The molecular genetics has been described in many of the neurodegenerative disorders, but many cases have been described with no single genetic cause found, even on whole exome sequencing. One of the common features though in many types of ND disorders has been the finding of up-and down-regulated genes in different areas of CNS, found on microarray studies, and called the common transcriptional signature of neurodegeneration. The common themes in these gene groups were genes belonging to neuroinflammation, mitochondrial dysfunction, protein aggregation, and metallothioneins.

1.2 Migraines

Headache disorders are a public-health concern given the large amount of associated disability and financial costs to society. Among adults of all ages, migraine is one of the top 20 causes of disability expressed as years of healthy life lost to disability according to the World Health Report 2001, WHO. Severe migraine attacks are classified by the World Health Organization as among the most disabling illnesses, comparable to neurodegenerative disorders, dementia, quadriplegia and active psychosis.

The definition of migraine according to the International Classification of Headache Disorders (ICDH-2) requires all the following:

a) recurrent headaches (at least 5 lifetime attacks)

b) untreated or unsuccessfully treated headache duration of 4-72 hours

c) at least two of the following pain characteristics: unilateral, pulsating, moderate or severe intensity, or aggravated by routine physical activity.

In addition migraines are associated with at least one: nausea/vomiting, photophobia, or phonophobia. Episodic migraines are characterized by 0-14 episodes per month, while chronic migraines occur on 15 or more days per month. There are many subtypes of migraines. Migraine with aura is associated with transient, and reversible focal neurologic symptoms that emerge prior to or during the emergence of the headache, involving mostly visual but other senses too. There are migraines without aura and migraine aura without headache according to the International Headache Society classification.
1.2.1 Familial hemiplegic migraine

Familial hemiplegic migraine (FHM) is a genetically heterogeneous autosomal dominant migraine with aura. FHM1 is caused by mutations in the \textit{CACNA1A} gene, encoding the pore-forming \(\alpha_1\) subunits of the neuronal voltage-gated \(\text{Ca}^{2+}\) channels; FHM2 is caused by mutations in \textit{ATP1A2}, encoding the \(\alpha_2\) subunit of the \(\text{Na}^+/,\text{K}^+\) ATPase; and FHM3 is caused by \textit{SCN1A}, encoding the pore-forming \(\alpha_1\) subunits of the neuronal voltage-gated \(\text{Na}^+\) channels\(^{13,14}\). In FHM the aura symptoms include uni- or bilateral motor weakness or paralysis, but the other symptoms are also present: visual, sensory, and aphasic. Some patients may also have severe attacks, with encephalopathy, coma and seizures. The hemiplegia can sometimes last several days. Emotional stress and minor head trauma have been shown to be triggers of episodes. FHM1 also can be associated with cerebellar symptoms, with or without nystagmus\(^{15}\). Two thirds of individuals with FHM experience cerebellar signs such as ataxia, and have cerebellar atrophy on brain imaging\(^{16}\).

Cerebral edema following minor head trauma has been described in many patients with FHM1. This phenotype has been most commonly associated with the Serine-218-Leucine (S218L) missense mutation in the \textit{CACNA1A} gene\(^{17,18}\), but several reports present with other mutations as well (i.e. T666M)\(^{19}\). Patients present with brain swelling after minor head trauma, preceded by a short lucid period, and sometimes the coma is fatal. The brain pathology in these cases reveals multiple infarcts in the cortex and basal ganglia, and swelling of the dendrites and widespread loss of Purkinje cells in the cerebellum\(^{17}\). MRI imaging reveals cortical and midbrain swelling and white matter T2 signal changes, with evidence of volume loss after recovery. The duration of coma can be several weeks\(^{20}\). There have been reports of patients with FHM who sustained irreversible brain damage after an episode of coma, with evidence of long-lasting MRI changes. This might be caused by initial cortical hypoperfusion, and eventually leads to cortical atrophy\(^{21}\). Ataxia with cerebellar atrophy and coma have been also described in patients with FHM2, with the \(\text{Na}^+/\text{K}^+\)-ATPase mutations\(^{22}\). The underlying cause of these mutations have been shown to be gain-of-function effect leading to ionic leakage\(^{23}\).

1.2.2 Migraine with aura

Migraine with aura and white matter abnormalities have been described in families with autosomal dominant cerebral arteriopathy with subcortical infarcts and leukoencephalopathy
(CADASIL). In rare cases, sporadic hemiplegic migraine can be the presenting symptom. CADASIL is caused by mutations in the \textit{NOTCH3} gene.

The disease will progress by middle age to a cerebrovascular disease manifesting as recurrent strokes, leading to dementia, and psychiatric manifestations. It is associated with white matter hyperintensities on MRI, with or without lacunar infarctions and microbleeds. One explanation for the migraine in CADASIL is cerebral hypoperfusion and ischemia, the other one is that CADASIL increases susceptibility to cortical spreading depression. The \textit{NOTCH3} gene encodes a transmembrane receptor present on the vascular muscle cells. The Notch signaling pathway is important in neuroglial development and for neural stem cell regulation. It has also been found to have an important role in the postnatal brain developmental process, and brain tumourigenesis. Episodes of reversible acute encephalopathy have been associated with CADASIL. In a series of 70 British patients with CADASIL six presented with coma. The episode lasted for several days up to two weeks, presenting with fever, confusion and seizures. All cases had previous history of migraine with aura and all cases recovered fully, but two of them had recurrent episodes over the years. There has been no clear understanding if loss-of-function or gain-of-function mutations cause CADASIL, but experiments looking at the distribution pattern of Notch receptors suggested a gain-of-function mechanism. The most likely effect of the gain-of-function is a misfolded protein and formation of aggregates. On immunohistochemistry there is increased Notch3 immunoreactivity in the brain vasculature with osmiophylic deposits.

1.2.3 Cortical spreading depression

Cortical spreading depression (CSD) is a slowly propagating wave of neuronal and glial cell membrane depolarization over the cortex that is the most likely explanation of the migraine aura. The phenomenon of “spreading depression” was initially described by Leao in 1944. During experiments on rabbits’ exposed brain, he found that shortly after the stimulation of a brain region, the spontaneous electrical activity decreased, then this decrease spread in all directions, affecting adjacent areas in succession. The depression was initiated more easily from the frontal regions. The recovery was usually slow, and happened also sequentially, taking 5 to 10 minutes in each region. Since Leao’s experiments, CSD has been studied extensively in different animal models. The initial depolarization of glial cells is accompanied by an increase
in extracellular $K^+$, a reduction in extracellular $Na^+$ and changes in other ions, such as $Cl^-$, $Mg^{2+}$, and $Zn^{2+}$, and this will lead to cellular swelling and shrinkage of extracellular space. Propagation of the CSD is nonsynaptic and the hypothesis is that it does spread through activity of a large astrocytic network. Further research in migraine patients led to the widely accepted hypothesis that the visual aura in migraine is caused by CSD. CSD is also associated with changes of the calibre of blood vessels, starting with an initial vasoconstriction that may propagate ahead of the CSD wavefront, then followed by a significant vasoconstriction. This is followed by a short recovery, then a sustained vasoconstriction that can last for hours, causing hypoperfusion. Functional imaging studies in FHM patients show propagation of oligemia followed by hyperemia, in a pattern of the CSD described in animal experiments $^{30,31}$. In FHM1 with gain of function mutations there is enhanced cortical excitability because of the increased glutamate release and enhancement of postsynaptic depolarization $^{32}$.

1.2.4 Migraine treatment

The prevalence of migraine headaches in North America is estimated at 18% in women and 6% in men. Considering the significant health care and financial burden of migraine sufferers, preventative medication has been introduced. About 3%–13% of identified migraine patients are on preventive therapy, while an estimated 38% actually need a preventive agent. The American Migraine Prevalence and Prevention Study outlined recommendations as to when daily pharmacological treatment should be initiated: at least six headache days per month; at least four headache days with at least some impairment; and at least three headache days with severe impairment or requiring bed rest. Medication should be started at a low dose, given each preventive medication an adequate trial, at least 2 months, avoiding interfering, contraindicated, or overused medications, and reevaluating therapy $^{33}$. Preventive medication mechanism of action includes inhibiting CSD through a variety of mechanisms, such as blocking calcium and sodium channels, enhancing the effect of the neurotransmitter GABA, having anti-adrenergic or serotonin modulatory effect. Examples of these are antiepileptics (i.e. topiramate, sodium valproate), beta-blockers and triptans. There are some vitamins that can also be used, such as riboflavin and coenzyme Q10 for prevention, but these have not been shown to inhibit CSD $^{33}$.

Out of the most commonly used migraine prevention drugs lamotrigine and to a lesser extent valproate have been shown to suppress CSD, but riboflavin had no effect $^{34}$. Intranasal
ketamine has been also looked at as a potential agent to stop the aura in FHM, hence the CSD. First it has been shown to block CSD in rats by acting on ion-gated channels and stabilization of postsynaptic membranes\textsuperscript{35}. In one study 5 out of 11 patients with severe FHM receiving 25 mg intranasal ketamine had reduced severity and duration of the neurologic deficits\textsuperscript{36}.

1.3 Neuroanatomy

Understanding the central nervous system (CNS) structure is the basis for learning neural function. Knowledge of the functional components, the neural connections, tracts and pathways allows one to formulate a differential diagnosis in neurological disorders, and also helps in designing specific therapies. This is true also for the knowledge of embryology and neural tube development.

1.3.1 Dorsal column-medial meniscus system

The dorsal column medial meniscus system fibers convey input from the periphery, and it goes rostrally toward the medulla and mesencephalic-diencephalic junction. In the caudal medulla they connect to their specific nuclei. In the spinal cord fibers traverse to the other side through the sensory decussation. The major neurotransmitters (NT) in this pathway are acetylcholine, glutamate and aspartate. Damage to the dorsal column fibers on one side of the spinal cord results in ipsilateral loss of vibration, position sense, and discriminative touch below the level of the lesion. Bilateral damage causes bilateral losses. Damage below the sensory decussation causes contralateral losses\textsuperscript{37}.

1.3.2 Anterolateral system

The anterolateral system terminates in the reticular formation (spinoreticular), deep layers of the superior colliculus (spino-tectal), the periaqueductal grey and midbrain reticular formation (spinomesencephalic) as well as thalamic nuclei (spinothalamic). Glutamate is the major NT in the dorsal root ganglion cells. Neurons in the periaqueductal grey and nucleus raphe dorsalis contain serotonin and neurotensin. Serotonergic raphespinal fibers may inhibit primary sensory fibers, conveying nociceptive information. Spinal lesions involving the anterior system result in loss of pain and temperature sensations on the contralateral side of the body beginning at the level of the lesion\textsuperscript{37}.

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1.3.3 Corticospinal tracts

The corticospinal tracts originate in the cortex and traverse the internal capsule to the other side below the pyramid (pyramidal decussation). Acetylcholine, GABA, and some peptides function as local circuits in the cortico-cortical connections. Glutamate is present in the fibers projecting to the spinal cord. Acetylcholine is also present in the lower motor neurons influenced by the corticospinal tract. Injury to the corticospinal tract on one side of the cervical spinal cord results in weakness or paralysis of the ipsilateral arm and leg. Patients also have upper motor neuron lesion signs: hyperreflexia, spasticity. Rostral to the pyramidal decussation, lesions in the medulla and pons or midbrain cause alternative hemiplegia, with contralateral hemiplegia of the arm and leg, and ipsilateral paralysis of the tongue, facial muscles, and most of the eye movements. Lesions in the internal capsule cause contralateral hemiparesis and corticobulbar involvement. Bilateral weakness is present in Amyotrophic Lateral Sclerosis (ALS) (Figure 1 and Figure 2).

Figure 1. Ascending and descending tracts in the midbrain
Image from Online Anatomy Course University of Western Ontario, London, Canada (Anatomical Foundations of Neuroscience) http://instruct.uwo.ca/anatomy/530/anfound.htm
1.4 Neurotransmitters

Neurotransmitters (NT) are chemical messengers that are released into the synaptic cleft upon the electrical impulse reaching the axon terminals. There are many categories of neurotransmitters: amino acids (glutamic acid, GABA, aspartic acid, glycine), peptides (vasopressin, somatostatin, neurotensin), monoamines (norepinephrine, dopamine, serotonin, melatonin, histamine), acetylcholine, and purines (adenosine, ATP, GTP and their derivatives). Each of these neurotransmitters will act on specific postsynaptic receptors. They can be categorized further into excitatory and inhibitory NT, according to their action on these receptors. Glutamate, an excitatory NT, is used in over 90% of the synapses; GABA is mostly inhibitory, and is used in more than 90% of the synapses that don't use glutamate; for dopamine the receptor determines if it is excitatory or inhibitory, monoamines and acetylcholine have modulating effect, and peptides have specialized functions in hypothalamus.

Monoamines are also called biogenic amines, and include serotonin, dopamine and norepinephrine. They have roles in modulation of psychomotor function, hormone secretion, cardiovascular, respiratory, gastrointestinal control, sleep mechanism, body temperature, and pain sensation regulation amongst others.
Knowledge of the NT accompanying each pathway is also important when we try to link the structural damage in the CNS to the symptoms in a patient with neurological presentation (Figure 3).

Figure 3. Serotonergic neurons and their projections in the brain

1.4.1 Neurotransmitter disorders

There are well known and described inherited disorders affecting neurotransmitter synthesis from aromatic amino acids that cause dopamine and serotonin deficiency. This group of disorders includes the cofactor, tetrahydrobiopterin (BH4) synthesis and recycling defects, tyrosine hydroxylase deficiency and aromatic L-amino acid decarboxylase deficiency. The BH4 synthesis defects are: autosomal recessive and dominant GTP cyclohydrolase (GTPCH) deficiency, the first enzymatic step in BH4 synthesis; 6-pyruvoyltetrahydropterin synthase (PTPS) deficiency; dihydropteridine reductase (DHPR) deficiency; and pterin α-carbinolamine dehydratase deficiency. These disorders with the exception of autosomal dominant GTPCH deficiency cause also hyperphenylalaninemia, because BH4 is also a cofactor for the phenylalanine hydroxylase enzyme. Tyrosine hydroxylase is the rate-limiting enzyme in
catecholamine synthesis and its deficiency causes a wide range of clinical symptoms varying between dopa-responsive dystonia to a severe progressive neurometabolic disorder characterized by a severe dystonic, dyskinetic movement disorder. Aromatic L-amino acid decarboxylase (AADC) deficiency is usually a severe, difficult to treat neurometabolic disorder, causing hypotonia, irritability, dystonia, oculogyric crises, myoclonic jerks, and autonomic dysfunction. Diagnosis of these disorders is made by measuring the following metabolites in the CSF: BH₄, BH₂, neopterin, sepiapterin, and the metabolites for dopamine (HVA, 3OMD), and serotonin (5HIAA). There is a well recognizable pattern of these metabolites to differentiate between these primary neurotransmitter disorders, for example in BH₄ synthesis or recycling defects both dopamine and serotonin metabolites will be low, because BH₄ is a cofactor for both tyrosine hydroxylase and tryptophan hydroxylase. This is the same in AADC deficiency, where the final step in the dopamine and serotonin synthesis pathway is blocked. In AADC deficiency the accumulating L-dopa will lead to elevated levels of 3OMD (3-O-methyldopa, metabolite of L-dopa). In tyrosine hydroxylase deficiency only the dopamine metabolite, HVA will be low. There is no isolated tryptophan hydroxylase deficiency described yet in human. The metabolic pathway involving biopterin and monoamine synthesis is illustrated in Figure 4.
1.4.2 Serotonin

Serotonin in the brain is stored inside vesicles in the neurons bound to serotonin binding protein (SBP). SBP is synthesized in the cell bodies of serotonergic neurons and transported down the axons to be stored inside the vesicles\(^\text{39}\). Serotonin then is released by exocytosis at the presynaptic terminal from synaptic vesicles, which is facilitated by Ca\(^{2+}\). After exocytosis, synaptic vesicles undergo endocytosis, recycling, and they refill with neurotransmitter\(^\text{40}\). Serotonin in the synapse will stimulate the various serotonin receptors then will be re-uptaken to presynaptic neuron by the serotonin transporter. Part of the serotonin will be stored in the vesicles, and part will go through enzymatic degradation by the mitochondrial enzyme monoamine oxidase (MAO) and form 5HIAA. Serotonin is released from intraneuronal vesicles in response to neural activity. There are two monoamine transporters VMAT1, expressed in the adrenal chromaffin cells and VMAT2 expressed in neural cells\(^\text{41}\). VMAT1 and 2 deficiency cause both serotonin and dopamine deficiency, and the clinical phenotype of its deficiency has been recently described\(^\text{42}\). The clinical presentation is with infantile-onset movement disorder (including severe parkinsonism and nonambulation), mood disturbance, autonomic instability, and developmental delay. (Figure 5).

Serotonin in the periphery is stored in platelets. Aggregating platelets release a number of vasoactive materials, including serotonin, which evokes vasoconstrictor response\(^\text{43}\).
Serotonin’s role in the body is multiple. It has been implicated in many physiological and behavioural functions in the central nervous system, such as sleep, appetite, vomiting, body temperature, sexuality, mood and aggression, but also in learning and memory\textsuperscript{44}. In addition animal studies have proven serotonin’s role in neurodevelopment, modulating the processes of neurogenesis, axon branching, and dendritogenesis\textsuperscript{45}. Although most of the early studies have been done on serotonin’s role in neuropsychiatric disorders, specifically in anxiety, and major depression\textsuperscript{46-48} there has been significant work done on the serotonin and gastrointestinal, cardiovascular, endocrine, and autonomic nervous system connection as well as pain/analgesia and breast cancer\textsuperscript{49-59}.

The number of receptors for the serotonin neurotransmitter system are the largest amongst all monoamines. There are 15 receptors identified that have been grouped in 7 families: 5-HT\textsubscript{1,2,4,5,6,7} predominantly coupled to G-proteins, and only the 5-HT\textsubscript{3} receptor being an ion channel.
Most of the G-protein coupled serotonin receptors act to inhibit cyclic AMP formation, open potassium channels, or increase phosphatidyl inositol (IP₃) hydrolysis. 5HT₁A receptors have been also shown to activate ERK (extracellular signal-regulated kinase) and Akt also known as protein kinase B, PKB, a serine/threonine specific protein kinase), thus inducing neuroplasticity. ERK signaling will eventually lead to gene transcriptional modulation (Figure 6).

![Diagram of serotonin receptor subtypes](image)

**Figure 6.** Interacting signaling pathways for 5-HT receptor subtypes (adapted from Aghajanian) MAP kinase: mitogen activated protein kinases, cGMP: cyclic guanylate monophosphate, Arf: ADP ribosylation factor, Arc: activity-regulated cytoskeleton-associated protein, C-fos: proto-oncogene, PLA: phospholipase A2, PLD: phospholipase D, PLC: phospholipase C, PKC: protein kinase C

### 1.4.3 Serotonin transporter

Serotonin is transported by the same transporter both in brain and platelets. In neurons, neurotransmitters are stored in vesicles, and after the vesicle fusion to the presynaptic membrane occurs, the neurotransmitters are released into the synaptic cleft, where they will activate pre- and post-synaptic receptors. Termination of synaptic signaling is terminated with the action of
the neurotransmitter transporter back into the neuron. Transport of serotonin is facilitated by the family of Na\(^+\) and Cl\(^-\) -dependent plasma membrane transporters, SLC6, the same family as the transporters of GABA, norepinephrine, dopamine, glycine, taurine, L-proline, creatine, and betaine. These transporter proteins are Na\(^+\) cotransporters, with the energy necessary for transport of the neurotransmitter provided by the electrochemical gradient for Na\(^+\). Cl\(^-\) is also cotransported but Na\(^+\) is the absolute prerequisite. Serotonin transporter SLC6A4 (SERT) also requires countertransport of K\(^+\). The gene for the transporter has 630 amino acids and 12 transmembrane domains. SERT activity has been implicated in various functions: mood, appetite, aggression, motor activity, sleep, and cognition. SERT knock-out mice have been extensively characterized. Serotonin concentration in extracellular compartment increases sixfold, and intracellular concentration decreases by 60-80\% \(^{64}\). Another consequence of absent SERT is down-regulation of 5HT\(_{1A}\) and 5HT\(_{2A}\) receptors\(^{65}\). SERT -/- mice have reduced 5HT re-uptake and high extracellular 5HT levels. There is no difference in TPH activity \textit{in vitro}, but there is increased \textit{in vivo} activity. 5HT synthesis is negatively controlled by presynaptic 5HT\(_{1B}\) and 5HT\(_{1A}\) receptors, which are desensitized in the SERT knock-out mouse. There is an increased 5HIAA/5HT ratio, indicating increased 5HT turnover rate, and there is less 5HT available for neurotransmission \(^{46, 66-68}\). SERT mRNA in brain colocalizes with tryptophan hydroxylase in serotoninergic cell bodies, in the dorsal raphe nuclei, and in lower concentration in the dorsomedial nucleus of the hypothalamus and cortex \(^{60}\). Genetic variants of SERT have been implicated in several neuropsychiatric disorders, such as Obsessive Compulsive Disorder (OCD), major depression, bipolar disorder, anxiety, and even associated with migraine with aura, and irritable bowel syndrome \(^{69-74}\).

In the human SERT gene promoter there is a common polymorphism, a 44-base pair insertion/deletion of a repetitive sequence \(^{46, 75} \). It is located approximately 1 kb upstream of the SERT gene transcription initiation site. The dominant short (s) allelic variant reduces transcriptional efficiency compared with the long (l) variant, leading to lower SERT mRNA levels, and a 40% decrease in serotonin re-uptake in platelets, affecting the transporter’s $V_{\text{max}}$ \(^{66, 67}\). Another regulatory variant is a 17 base-pair variable tandem repeat (VNTR-17) located in intron 2, with two common (10 or 12 repeats) and one rare (9 repeats) allele. These tandem repeats seem to modulate the gene’s transcription in allele dependent manner, i.e. higher number of repeats correspond to higher gene expression (transcriptional enhancer) \(^{46}\).
1.4.4 Serotonin in treatment

Serotonergic neuronal projections are present throughout the spinal cord, mainly in the motor neural area of the ventral horn, and in the cerebral cortex. Various types of serotonin receptors are also present in the spinal cord, and medulla oblongata, with specific distribution. Rat spinal cord mRNA studies showed that in contrast to 5HT$_{2C}$ receptors being widely distributed throughout the ventral and dorsal horns of the spinal cord, 5HT$_{2A}$ receptors are exclusively located in lamina IX of the ventral horn. The medulla had abundant 5HT$_{2C}$ receptors, which were widely distributed, but less 5HT$_{2A}$ receptors. This difference in distribution helps to understand their role in the mechanism of motor function and nociception. Serotonin has been shown since the 1970’s to be a potent modulator of motor neuron excitability in the spinal cord, through its effect on the glutamatergic system. Numerous animal studies have been conducted in relevance to developing a therapy for motor neuron disorders (MND). Experiments conducted on rats with complete spinal transection at the T5 level, and given intraperitoneal 5HT precursor, 5-hydroxytryptophan (5HTP), showed increased excitability of motor neurons located below the transection, measured by spontaneous EMG activity in the paralyzed hindlegs. In another experiment on superficial dorsal horn cells in rat spinal cord slices, 5HT was found to produce long-lasting presynaptic facilitation (mediated by the 5HT$_2$ receptor linked to protein kinase C), as well as a transient inhibition of glutamatergic excitatory postsynaptic currents (mediated by the 5HT$_1$ receptor). One of the hypotheses explaining the action of 5HT on spinal neurons is hyperpolarization of the membrane potential, which facilitates neuronal recruitment and firing. Others explain the spinal cord motoneuron excitability with the role of 5HT in regulation of calcium currents. In the brain, 5HT together with the brain-derived neurotrophic factor (BDNF) are known to regulate synaptic plasticity, neurogenesis, neurite outgrowth, neuronal protection and survival, and if this is compromised, could lead to neurodegenerative disorders such as Alzheimer’s and other age-related diseases. 5HT also has been shown to have neuroendocrine roles, such as stimulating the secretion of prolactin, growth hormone, and ACTH directly at pituitary level, probably by releasing a mediator or mediated through the action of the vasoactive intestinal peptide.

Serotonin has an important role in modulation of synaptic transmission. Serotonin has been shown to inhibit presynaptic glycinergic synaptic transmission. This is explained with two mechanisms: through modulation of K$^+$ and voltage-gated Ca$^{2+}$ channels. 5HT potentiates
inwardly K\(^+\) channels and attenuates voltage-activated Ca\(^{2+}\) channels through activation of 5HT\(_{1A}\) receptors. 5HT\(_{1B}\) receptors are also distributed in the presynaptic terminal and are coupled to G-proteins. Their activation inhibits accumulation of cAMP, mediating directly presynaptic inhibition. This means that 5HT inhibition of glycinergic transmission is two-fold, indirect through ion channels and direct through inhibition of synaptic transmission. Glycine is the major inhibitory neurotransmitter in the spinal cord and brainstem, and modulating glycinergic transmission might explain the motor enhancement in treatment with 5HT.

Serotonin is also a modulator of glutamate- and GABA-mediated neurotransmission\(^{86}\). In many brain regions 5HT induces a decrease of glutamate and increase of GABA transmission. This is also mediated through the various 5HT receptors. In other brain regions, especially the ones participating in motor control, such as in the spinal cord, 5HT can enhance glutamate- and decrease GABA- mediated transmission. This is mediated often through NMDA receptors that can be normalized in case of hypofunction by administration of 5HT antagonists. Hyposerotoninemia has been also linked to Parkinson’s disease, by finding reduced levels of serotonin binding sites and transporters in the striatum. Administration of serotonin re-uptake inhibitors not only improves the depressive symptoms, but also the bradykinesia. This is thought to be GABA-mediated response to 5HT. Serotonin-glutamate interactions also play a role in the pathogenesis of migraine. 5HT receptor agonists modulate glutamate release and decrease the NMDA-induced NO synthesis, which is the main factor in triggering the headache\(^{80}\).

In the dorsal horn of the spinal cord serotonin potentiates GABA\(_A\) and glycine receptor-mediated responses through G-protein-coupled 5HT receptor mediated responses, mainly through protein kinase C (PKC). In isolated spinal cord serotonin also reduces propagation of the sensory signals through reduction of excitatory postsynaptic potentials.\(^{87}\)

5-hydroxytryptophan (5HTP) is the intermediate metabolite of L-tryptophan in the synthesis of serotonin. It is absorbed readily in the gut, and does not participate in formation of niacin or protein like L-tryptophan. It also crosses easily the blood-brain-barrier, and increases the serotonin synthesis in the CNS\(^{88}\). Administration of 5HTP together with carbidopa, a peripheral aromatic L-amino acid decarboxylase inhibitor increases significantly the plasma and CSF concentration of 5HT\(^{89}\). Administration of 5HTP to transgenic mice overexpressing wild-type SOD1, which models the human amyotrophic lateral sclerosis (ALS), significantly delayed development of hindlimb weakness and mortality in a dose dependent manner. Improvements in muscle tone, posture, and motor function, and increased blood serotonin levels were noted. This
again suggests that serotonin has a role in neuromodulation in motor neuron disease. Serotonin has been also found to be one of the disease modifying factors in ALS. A large study of 85 patients with ALS done by Dupuis et al. demonstrated a 30% decrease of platelet serotonin levels compared to controls. There was also a significant decrease of serotonin levels with increasing age in ALS patients. Statistical analysis showed a 57% decreased risk of death for patients with platelet serotonin levels in the normal range relative to patients with abnormally low platelet serotonin. This positive correlation between platelets serotonin levels and survival suggests once again that serotonin has an important role in motor neuron disease, and opens new perspectives for their treatment.

Ramaekers et al. described five patients with hypotonia, motor delay and learning disabilities who had low CSF and urinary serotonin metabolite, 5HIAA. No molecular defect was found in these patients, but all five of them responded with clinical improvement and normalization of the biochemical parameters to administration of 5-hydroxytryptophan and carbidopa.

1.4.5 Serotonin synthesis and metabolism

Serotonin (5HT) is synthesized from tryptophan through the rate limiting enzyme tryptophan hydroxylase (TPH) in the presence of the cofactor tetrahydrobiopterin (BH4). This first step produces 5-hydroxytryptophan, which in turn will be decarboxylated by the enzyme aromatic L-amino acid decarboxylase into serotonin. Tryptophan is one of the nine essential amino acids that cannot be synthesized by humans, hence needs to be obtained from external sources. The synthesis of serotonin is only a minor route for tryptophan, 95% of tryptophan will be catabolized onto the kynurenine pathway. The final product of this pathway is nicotinamide adenosine dinucleotide (NAD), with kynurenine the first stable intermediary metabolite. From kynurenine several neuroactive metabolites such as 3-hydroxyanthracillic acid, quinolinic acid and kynurenic acid are generated, which have either excitotoxic (3-hydroxyanthranilic acid, quinolinic acid) or neuroprotective properties (kynurenic acid, picolinic acid) (Figure 7). The kynurenine pathway is upregulated in inflammatory processes, when the immune response is activated. The kynurenine pathway has been shown to be involved in many human diseases: Alzheimer disease, ALS, MS, Huntington’s disease, schizophrenia, AIDS.
dementia complex, and others, where a higher ratio of kynurenine/tryptophan was observed, especially in advanced disease state (Figure 7).

Figure 7. Tryptophan - serotonin pathway

In the brain 5HT is synthesized in the raphe nuclei in the medulla, in serotonergic neurons, and in the periphery in the gut enterochromaffin cells. 95% of the body total serotonin is contained in the enterochromaffin cells, and other important tissues that contain serotonin are the brain, mainly the medulla, the pineal gland, and platelets. However platelets do not synthesize serotonin. Instead it originates in the enterochromaffin cells in the gut. After synthesis, serotonin is released into the gastrointestinal lumen, and then after crossing the basolateral membrane, it will end up in the portal venous blood. In blood serotonin almost immediately will be taken up by platelets, and very little will be found in plasma. The other two ways of elimination/degradation of serotonin is metabolism by the liver and the lungs. The enzyme tryptophan hydroxylase is present in most tissues containing serotonin, except in platelets. In most tissues TPH is the rate-limiting step in serotonin synthesis. The K_m of TPH in brain for tryptophan is relatively high (3 x 10^{-5} M). In normal circumstances the concentration of
tryptophan in brain is lower than this, so the enzyme is not saturated. The rate of the serotonin synthesis is controlled by the availability of the amino acid tryptophan\textsuperscript{95}. The transport of tryptophan through the blood brain barrier is done through an affinity based L-type neutral amino acid transporter. 80 to 90% of the plasma tryptophan is bound to albumin and this transporter will strip it off the albumin\textsuperscript{96}. Binding of tryptophan to albumin also depends on the available circulating free fatty acids amount. Tryptophan is in competition with the other neutral amino acids (phenylalanine, leucine, tyrosine, methionine, histidine, isoleucine, valine, threonine) for the transporter binding sites\textsuperscript{97}. The presence of large neutral amino acids in the plasma is limiting the tryptophan uptake into the brain.

There are two types of tryptophan hydroxylase enzymes: Tryptophan hydroxylase 1 (TPH1), controls serotonin synthesis in the periphery, and Tryptophan hydroxylase 2 (TPH2), which controls serotonin synthesis in the brain\textsuperscript{98}. The two different enzymes are encoded by two different genes: \textit{TPH1} and \textit{TPH2}. \textit{TPH} mRNA quantification in rat raphe and pineal glands also confirmed an anatomical expression pattern for \textit{TPH2} in the raphe and for \textit{TPH1} exclusively in the pineal gland\textsuperscript{99}. There has been a growing body of research looking into linking neuropsychiatric disorders with serotonin metabolism, and hence linking functional polymorphisms in the \textit{TPH2} gene with different neuropsychiatric disorders, including major depression\textsuperscript{100}.

1.4.6 Serotonin developmental pathway

Serotonergic projections to cortical regions come from the dorsal and median raphe nuclei of the brainstem. Several regulatory genes contribute to the specification, differentiation and phenotype maintenance of the raphe serotonergic system. Fibroblast growth factors (Fgf8), “Sonic hedgehog” (Shh), Gli2, and Pet1 signals control serotonergic cell fate. Amongst these Pet1 and GATA3 have a critical regulator role of the serotonergic system specification. SPT5 or Foggy (\textit{SUPT5H}), a phosphorylation-dependent dual regulator of transcript elongation also affects development of serotonin containing neurons\textsuperscript{101}. It is essential for embryo survival, and gene expression\textsuperscript{102}. Foggy mutant zebrafish had reduced dopaminergic and increased serotonergic neurons in the hypothalamus\textsuperscript{103}. One of the down-regulated target genes of SPT5 in zebrafish is the Na\textsuperscript{+}/K\textsuperscript{+} ATPase α1a.4\textsuperscript{104}. Transcription of RNA is controlled by a variety of
initiation and elongation elements. SPT5 together with SPT4 regulate transcriptional elongation by RNA polymerase II (Figure 8).

**Figure 8.** Transcription factors
(adapted from Lesch) **Transcription factors:** Wnt1, Pax2, Pax5, Otx2, Gbx2, En1, En2, Shh, Fgf8, Glx1, Pet1, SPT5, Eg, and GATA3. **Neurotrophins:** BMP (bone morphogenetic protein), TGFβ (transforming growth factor β), CNTF (ciliary neurotrophic factor), BDNF (brain-derived neurotrophic factor), TrkB (neurotrophin receptor), MAPK (MAP kinase), AD (adenyl cyclase), PKA (protein kinase A), TPH (tryptophan hydroxylase), R (receptor).

Several genes in this pathway have been already associated with human disease. GATA3 mutations have been linked to hypoparathyroidism, sensorineural deafness and renal anomalies (HDR) syndrome\textsuperscript{105}. Mutations in this gene have also been found in breast tumours\textsuperscript{106}. The brain-derived neurotrophic factor (BDNF) has been suggested as candidate gene in a number of neurodegenerative and psychiatric disorders, such as Alzheimer’s and Parkinson’s disease, bipolar disorder and schizophrenia\textsuperscript{107-110}. Lmx1b gene has been shown to have a role in renal disease development in the nail-patella syndrome\textsuperscript{111}. 

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The PET1 or FEV (Fifth Ewing variant) gene has been found to play a critical role in serotonergic neuron development and a rare variant was found to be common in SIDS related deaths in the African-American population\textsuperscript{112-114}.

1.5 Cytoskeleton

Gearing \textit{et al.} described in 2002 aggregation of actin and coflin in identical twins with juvenile onset dopa-unresponsive dystonia\textsuperscript{115}. The authors found eosinophilic, rod-like cytoplasmic inclusions in neocortical and thalamic neurons that were actin depolymerizing factor (ADF)/cofilin immunoreactive, and similar, spherical structures in the striatum. This was the first report of actin aggregation in a neurodegenerative disorder, but no underlying genetic diagnosis was made in these patients. The twins had history of mild developmental delay until the age of 12 years, when they developed a rapidly progressing generalized dystonia, initially affecting their gait, and after a neurodegenerative course with end-stage cranial and bulbar involvement, they died in their early 20’s. The neuropathological finding of colocalizing actin depolymerizing factor (ADF)/cofilin aggregates suggest a dysfunction in the actin regulatory system. The authors hypothesized that the phosphorylation status of ADF/cofilin, determining their activity is somehow at the cause of the aggregation\textsuperscript{115}.

The actin cytoskeleton plays a major role in intracellular trafficking, and in the central nervous system controls the dynamic synaptic functions. The main cytoskeletal proteins involved are: F-actin, microtubules (filaments of tubulin), and fodrin (brain form of spectrin). Actin remodeling is influenced by actin binding proteins (ABP), such as gelsolin, coflin, profilin, and tropomodulin amongst others. These proteins not only help with the structural organization of the cytoskeleton, but are also targets for signal transduction molecules\textsuperscript{116}. Actin is an important component of the presynaptic terminal, it regulates the reserve pool of the synaptic vesicles, plays role in vesicle mobilization, delivering them to the active zone and promoting exocytosis, thus having direct role in neurotransmitter release into the synapse. By causing structural changes actin has role in short term and long term synaptic plasticity\textsuperscript{116}. Dynamic changes of the actin cytoskeleton, driven by the different downstream signal transduction cascade are at the base of cell motility also. Mutations in varied actin binding proteins and signaling molecules were found in a variety of immunological disorders and cancer\textsuperscript{117}. Actin based cell motility is also critical in the nervous system development. Actin polymerization controls membrane extension and retraction, modulating neurite growth, and
growth cone motility. It also regulates neuron migration and axonal guidance. Reorganization of actin cytoskeleton is modulated through rho GTPases, and their downstream effectors, Arp2/3 complex, ADF/cofilin, LIM kinases and Ena/VASP proteins amongst others\textsuperscript{118} (Figure 9).

![Diagram of actin remodeling](image)

**Figure 9.** Cytoskeletal elements (adapted from Dillon and Goda\textsuperscript{116})

The cytoskeletal actin exists in two forms, monomer G-actin and filament F-actin. These two forms are in equilibrium and depending on activity various kinases (PKC in particular), phosphatases, intracellular Ca\textsuperscript{2+} ions, c-AMP, ATP and other factors, one will be more prevalent than the other. The state of the actin cytoskeleton modulates the activity of Na\textsuperscript{+}/Cl\textsuperscript{-} dependent neurotransmitter transporters as shown by Sakai et al. in COS-7 cell experiments\textsuperscript{119}. The
authors demonstrated that inhibition of F-actin formation reduced the uptake activity of the serotonin transporter. One of the explanations they proposed was that the morphological change in the cell caused by disruption of actin filaments reduce the number of SERT expressed on the membrane, reducing the $V_{max}$ but not altering the $K_m$.

Most of the plasma membrane SERT is associated with the membrane cytoskeleton. This association can be enhanced by SERT or 5HT2A receptor activation. Inactivation of the serotonin transporter leads to internalization, and relocation of the transporter to the cytoskeletal fraction. Experiments with subcellular fractionation of platelets show that SERT is most abundant in the membrane skeleton fraction, followed by the Triton X-soluble fraction and only in very small amount in the cytoskeleton.

Other cytoskeleton components, such as ankyrin and spectrin, have important roles in transport of proteins to the cell membrane. Mice lacking certain isoforms of ankyrin will have the voltage-gated Na+ channel accumulated inside the sarcoplasmic reticulum of the cell, and mutations in spectrin will cause similar problems, involving other channels such as K+ or Ca^{2+} channels (i.e. in spinal cerebellar ataxia type 5) The $\alpha_1$-Na+-K+-ATPase binds directly to ankyrin, and disconnection from this bound form will lead to the ATPase being trapped inside the cell, instead of being transported to the cell membrane.

Minamide et al. proposed a common pathway leading to loss of synapses that is related to rod-like inclusion formation containing ADF/cofilin and actin. These rods are forming as a response to neurodegenerative stimuli, which induces activation (dephosphorylation) of ADF/cofilin.

Aggregation of cytoskeletal proteins can be seen in many familial neurodegenerative disorders, such as Amyotrophic Lateral Sclerosis (ALS), frontotemporal dementia with parkinsonism (FTD), sporadic progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD). The inclusions are broadly classified as filamentous aggregates of neuronal intermediate filament (IF) proteins or as microtubule-associated protein (MAP) tau. These aggregates can be seen on neuropathological examination and immunohistochemistry.

Changes in the neuronal cytoskeleton, caused by mutations in MAP, actin-binding proteins, and altered expression levels of several cytoskeleton proteins have also been described in epilepsy syndromes, for example $\alpha$-tubulin, doublecortin (DCX), lissencephaly-1 (LIS1), ankyrin-repeat containing protein (KRIT), filamin A, cofilin, gelsolin and actin-severing proteins.
Considering the many roles of the cytoskeleton, playing key roles in the development of the nervous system, functional brain plasticity, synaptogenesis, and neurotransmitter release, it is considered one of the favorite therapeutic target in neurodegenerative and some of the psychiatric disorders. Some studies looked at the role of acetylcholinesterase inhibitors’ role on modifying cytoskeleton architecture, others focused on the therapeutic action of antipsychotics. Administration of certain antipsychotics cause synaptic alterations, others interfere with the phosphorylation of the cytoskeletal-stabilizing protein MAP2, and some will inhibit axonal transport. One of the studies looked at the role of melatonin in cytoskeleton reorganization. Oxidative stress is the hallmark of disease in some dementias and mental disorders, and reactive oxygen species produce rearrangements in cytoskeleton. Melatonin is a potent free radical scavenger, and it is also acting as a cytoskeleton modulator.

1.6 Axonal transport

Axonal transport is essential for neuronal viability and function. It is the flow of proteins and organelle cargoes to and from the neuronal body. The anterograde transport takes molecules from neuronal cell body to the nerve terminal, supplying proteins and lipids and synaptic components, and the retrograde transport, from the terminal to the neuronal body, removes ageing proteins for degradation and recycling. A number of adult onset neurodegenerative diseases, including ALS, SMA, HSP, Charcot-Marie-Tooth (CMT), Alzheimer’s, Parkinson’s and Huntington’s diseases have been linked to dysfunction of axonal transport. There is fast and slow anterograde axonal transport, which is ATP dependent, and fast retrograde axonal transport, which is ATP independent. The molecular mechanisms underlying these disorders are classified into: dysfunction of molecular motors (kinesin and dynein), microtubules, cargoes, and mitochondria. The molecular motor transport occurs along the neuronal cytoskeleton, which consists of microtubules, actin filaments, and intermediate filaments. Microtubule-associated proteins (MAPs) promote microtubule stabilization, and regulate transport by modulating the interaction of molecular motors with microtubules. Kinesins are important motors driving the anterograde transport of a wide range of cargoes, including vesicles, organelles, and proteins. Cytoplasmic dyneins are the major retrograde transport motors. Dynein function requires activators, called dynactin. Both dyneins and kinesins are fast motors, with velocities at 0.5 – 1 μm/sec. Axonal proteins and synaptosomal components are transported by fast anterograde transport, and autophagosomes and signaling endosomes by retrograde transport. Mitochondria
and lysosome transport is bidirectional, and is regulated by neuronal activity. Cytosolic protein, such as actin and tubulin are transported through the slow axonal transport\textsuperscript{127, 128}. Axonal transport defects can be seen in microscopy studies, which show that cytoskeletal components can accumulate in the proximal or distal segment of the axon. In many neurodegenerative disorders the molecular defects have been described in a variety of genes such as: APP, PSEN, MAPT in Alzheimer’s disease and other dementias, SNCA, PARK2, PINK1, PARK7, DCNT1 in Parkinson’s disease and related syndromes, HTT in Huntington disease, ATL1, SPAST, NIPA1, KIF5A, ZFYVE26, KIAA0610, ACP33, REEP1, ZFYVE27 in Upper Motor Neuron disease, MFN2, TBC1D15 NEFL, HSBP1, DYNCIHI in CMT, DCTN1, AR in Lower Motor Neuron disease, and SOD1, ALS2, and VAPB in ALS\textsuperscript{129}.

Cytoplasmic dynein is a large complex protein composed of heavy chains, intermediate chains, light-intermediate chains and light chains. The cytoplasmic dynein heavy-chain protein encoded by DYNC1HI is an important member of axonal transport proteins. Dyn1h1 knock-out mice, if homozygous, die at birth, if heterozygous, show motor deficiencies and have evidence of loss of motor neurons, similar to ALS caused by SOD1 mutation\textsuperscript{130}. Axonemal dynein on the other hand, like DNAH3 are driving the ciliary and flagellar motility. This is expressed primarily in trachea and testes\textsuperscript{131}.

1.7 RNA processing and neurological disease

1.7.1 Pre-mRNA splicing and neurologic disease

In the late 1970s scientists demonstrated that eukaryotic genes contained sequences that were not present in mature mRNA. They called it precursor-messenger RNA (pre-mRNA)\textsuperscript{132}. The removal of introns from pre-mRNAs is performed by RNA splicing. It is carried out by a large RNA-protein complex called the spliceosome, that contains over 300 proteins and five small nuclear ribonucleoprotein particles, the U1, U2, U4, U5, and U6 snRNPs\textsuperscript{133-135}. Defects in RNA metabolism and splicing defects in particular, have been implicated in many diseases such as cancer, muscular dystrophy (Myotonic dystrophy, SMA) and most neurodegenerative diseases. (ALS, Frontotemporal Dementia and Parkinsonism, Parkinson’s disease and Alzheimer’s disease)\textsuperscript{136, 137}.
1.7.2 SR proteins

The SR proteins are part of the spliceosome complex, and they are proteins rich in arginine and serine dipeptide domains, termed the RS domain that bind to active sites of RNA polymerase II transcription. They were first discovered in the 1990 as splicing factors\textsuperscript{138,139}. They contain one or two copies of an RNA recognition domain (RRM) at the N-terminus, and a C-terminal RS domain that promotes protein-protein interactions, important in recruitment and assembly of the spliceosome. The RS domain is extensively phosphorylated on serine residues and this plays an important role in regulating the subcellular localization and activity of SR proteins\textsuperscript{140-142}. The SR-like or SR-related proteins contain an RS domain but lack a defined RRM, and they have roles not only in splicing but also in other cellular processes such as chromatin remodeling, transcription and cell cycle progression. The splicing coactivators SRm160/300 are such SR-related proteins, amongst others. Disruption of many roles of SR family proteins can lead to human disease. Approximately 15% of mutations that cause genetic disease affect pre-mRNA splicing. This number includes mis-splicing that are due to splicing machinery defects and mutations in the gene itself, such as in exon/intron junctions. Increased expression or mis-expression of SR proteins, resulting in alternative splicing of transcripts has been correlated with cancer and its progression, for example in malignant ovarian or breast cancer. In amyotrophic lateral sclerosis (ALS), Alzheimer disease (AD), and Parkinson’s disease (PD), gene expression profiling has revealed dysregulation of genes that are related to neuroinflammation. Also in ALS and PD, there have been many reports about disruption in gene expression related to RNA splicing. Several studies in ALS also support involvement of the cytoskeleton\textsuperscript{143}.

Genome-wide transcription analysis in Parkinson’s disease revealed that the pre-mRNA splicing gene \textit{SRRM2} (serine-arginine repetitive matrix 2) was the only gene differentially upregulated in PD patients compared to controls\textsuperscript{144}. The authors argued that this gene might have some role as a biomarker in PD.

Recent studies in AD indicate that aberration in the control of gene expression might contribute to the initiation and progression of the pathology of the disease. Transcriptomic analysis of distinct regions of AD brain using RNA-Seq next generation sequencing technology revealed differing expression levels of APOE-001 and 002 splice variants\textsuperscript{145}. 

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1.7.3 SRRM2

In 1998, Blencowe et al.\textsuperscript{146} described a novel serine and arginine rich protein associated with splicing complexes and named it SRm160 (SR-related matrix protein of 160 kD) also known as SRRM1. As the other SR-related proteins, SRm160 contains multiple SR repeats, but lacks an RNA recognition motif. Later research has shown that SRm160 forms a complex with another SR-related protein SRm300, or SRRM2. This complex associates with splicing complexes and promotes splicing through interactions with other SR family proteins, functioning as a co-activator of pre-mRNA splicing. The SRm160/300 complex is required for the purine-rich Exonic splicing enhancer (ESE) sequences to recognize splice sites in pre-mRNA. SRm300 contains a novel and highly conserved N-terminal domain, several unique repeated motifs rich in serine, arginine, and proline residues, and two very long polyserine tracts\textsuperscript{147}. The high content of SR dipeptide repeats and other repetitive sequence features suggest that it is capable of interacting simultaneously with many factors, including SRm160 and many other proteins. Such a function could be important for the organization of SR proteins and other splicing factors within the cell nucleus. The two very long polyserine tracts in SRm300 are reminiscent of serine-rich domains that have been identified in other nuclear proteins. Several kinases phosphorylate RS domains and are known to influence protein-protein interactions between SR proteins and splicing activity. These kinases could influence interactions in splicing involving SRm160/300\textsuperscript{148}. SRm300 is tightly bound to the nuclear matrix, and its phosphorylation could influence the nuclear distribution of SRm160 and/or other associated SR proteins, and thereby modulate splicing activity by regulating the availability of these proteins at the sites of nearby transcription (Figure 10).

![Figure 10. SRm300/SRm160 in the spliceosome complex (adapted from Blencowe 1998\textsuperscript{146})](image-url)
1.7.4 SRRM2 protein interactions

*HitPredict* is a web-based resource of high confidence protein-protein interactions. Interactions can be searched and downloaded with their predicted confidence level. Protein-protein interactions from IntAct, BIOGRID and HPRD are combined, annotated and assigned a reliability score in order to identify a high confidence subset.

The reliability score is calculated as the Likelihood Ratio using naive Bayesian networks combining sequence, structure and functional annotations of the interacting proteins. Interactions of SRRM2 protein are shown in Figure 11. There are 135 protein-protein interactions found from high throughput experiments with SRRM2 and the highest likelihood of interaction is with PININ (likelihood 999.00)

![Interaction Edge Legend](#)

**Figure 11. SRRM2 protein-protein interactions**

Interaction prediction was based on data from HitPredict, a database of high confidence protein-protein interactions (http://hintdb.hgc.jp/htp/)

1.7.5 Yeast SRRM2 homologue

Cwc21p, a 135 amino acid protein in *Saccharomyces cerevisiae*, a protein of previously unknown function, was found to share homology with the N-terminal domain of human SRm300/SRRM2, and was shown to be the yeast ortholog of the human SRRM2 protein.

A series of experiments have shown that it is associated with a group of proteins involved in activating the spliceosome to promote the pre-mRNA splicing reaction \(^\text{149}\). Genetic and functional studies on Cwc21p indicate that they have related functions at or prior to the first
catalytic step of splicing, and suggest that Cwc21p functions at the catalytic center of the spliceosome, possibly in response to environmental or metabolic changes. Alignment of the Cwc21p/SRm300 family shows a highly conserved region of around 100 amino acids at the N terminus of each protein, and has the following three features: 1) a conserved N-terminal motif of about 24 amino acids, 2) a variable linker region, and a 3) a conserved cwf21 domain, named after the cwf21, the *Schizosaccharomyces pombe* ortholog. Proteins of the Cwc21/SRm300 family are usually larger in higher eukaryocytes due to extended C-termini that can be 2000 or more amino acids long. These extensions are made up of poly-serine stretches and RS di-peptide repeats, the latter being known to direct protein-protein or protein-RNA interactions\(^\text{135, 150}\). Experiments suggest multiple functions for Cwc21/SRm300 in the splicing process\(^\text{149}\). Cwc21-\(\Delta\) strains (a viable strain deleted for cwc21) did not show observable growth defects, and intron-containing genes did not display any pronounced defects in pre-mRNA processing. However overall transcript levels were somewhat reduced for transcripts from intron-containing genes relative to transcripts from intronless genes in this strain. Tiling microarray analysis and subsequent RT-PCR assays of transcripts in this mutant strain did not reveal any aberrantly spliced products, only differences in the efficiency of splicing\(^\text{149}\).

While mammalian SRm300 shares significant similarity within its N-terminal domain with Cwc21, the mammalian protein contains a remarkably long C-terminal region that is highly enriched in alternating Arg/Ser dipeptide repeats (RS domains) and other repetitive features.

### 1.7.6 *Caenorhabditis elegans* ortholog

The *Caenorhabditis elegans* ortholog of the human spliceosomal protein SRm300/SRRM2 is RSR-2. Knockdown of the RSR-2 disrupts early development suggesting a possible critical, gene-specific role for this protein in splicing activity.

Inactivation of RSR-2 by double stranded RNA (dsRNA) microinjection produces larval-arrested animals\(^\text{151, 152}\). RSR-2 RNAi approaches by microinjection or by feeding (with a weaker effect) give rise to a variety of phenotypes, from larval and embryonic lethality to reduced brood size and sterility. These produce partial inactivation of RSR-2. The allele tm2607 produces a truncated protein that is 65 amino acids shorter. Although several arginine and serine residues were eliminated, the truncation did not affect the cwc21 motif (the highly conserved N-terminal region that shares similarities between yeast, worm and human), and the deleted region
turned out not to be essential for RSR-2 functions. Tiling microarrays showed that RSR-2(RNAi) L4 worms had slightly lower global transcript levels while splicing remained unaltered. There were several upregulated and downregulated genes. The list of downregulated genes was enriched in germline genes, particularly those involved in spermatogenesis. There were no significant variations in alternatively spliced transcripts. Immunopurification of RSR-2 containing complexes from wild-type worm population identified that RSR-2 interactors were components of the spliceosome and also proteins related to stress response, transcription, chromatin regulation, translation and ubiquinitation. These results together suggest that during development, mild RSR-2 deficiencies may primarily affect highly transcribed genes.

RSR-2 interacts with proteins related to splicing and transcription, amongst others PRP-8, SKP-1, KIN-3 and PRP19. SKP-1 is the ortholog of SKIP/SWN1 in humans and it plays a role in splicing and transcription elongation. KIN-3 encodes the ortholog of human catalytic subunit of CKII, a protein kinase that phosphorylates several substrates regulating transcription and interacts with splicing factor PRPF3. PRP-19 is the ortholog of human PRPF19, involved in pre-mRNA processing and transcriptional efficiency. Proteomic analysis of RSR-2 containing complexes support the hypothesis that RSR-2 influences the transcriptional machinery through protein-protein interactions.

Proteomic analysis of yeast Cwc21 and C. elegans RSR-2 interactions need to be expanded in human SRRM2, as it is a much larger gene. Changes in the gene structure especially in the highly repetitive SR region might affect interaction with other spliceosomal protein components.

1.8 Whole exome sequencing

To date the majority of mutations identified in Mendelian diseases have been located in the 1% of the human genome, or the coding or exon regions. The technique of Exome sequencing targets these regions and has been proven to be successful in identifying novel causative mutations in genetic disorders. This method starts with the shearing of genomic DNA into random fragments, then enriching the target fragments in exome regions using oligonucleotide probes, to hybridize these target sequences. The three major commercial next-generation sequencing platforms are by Illumina, Roche, and Applied Biosystems. Depending on the technology used, an individual exome is found to have 20-30,000 variants. Of these, about 10,000 will be non-synonymous amino acid substitutions, conserved splice site alterations or small insertions and deletions. Up to 90% of these variants can be found in publicly available
databases, such as dbSNP and 1000 Genome Project, and if commonly found they can be regarded as polymorphisms. Other variants can be filtered out if they are predicted to be benign or tolerated by computational algorithms. Two of these predicting tools are Sift and Polyphen ([http://sift.jcvi.org/](http://sift.jcvi.org/), [http://genetics.bwh.harvard.edu/pph2/](http://genetics.bwh.harvard.edu/pph2/)). To further decrease the number of candidate genes, family trios are used, with addition of parental samples, and further addition of affected and unaffected siblings have even higher chance of detecting the genes responsible for the clinical phenotype.

1.9 RNA sequencing (transcriptome analysis)

RNA sequencing is a new technique to analyze global patterns of gene expression in an organism. It is a high-throughput sequencing technology for characterizing RNA content in a given sample. The advantage over the previously used RNA microarray methods is that it can also provide information on splicing events. Similar to whole exome sequencing various sequencing platforms can be used for bioinformatics analysis.

Data analysis can be done with GeneSifter (Analysis Edition), a statistical, visualization and annotation tool that was designed for Microarray and Next Generation Sequencing data analysis. With this software the user can define the statistical analysis to compare multiple experiments using pairwise analysis or ANOVA. It integrates KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) lists to give a more comprehensive view of which pathways and ontology groups are differentially regulated. Fold change and pathway analysis are totally integrated. Once the list of genes with statistically significant changes in regulation is generated, the user can get more detailed information on all genes. The charts and graphs make the data much easier to understand.
Chapter 2: Case presentation

2.1 Introduction

Ten years ago a 15-year-old adolescent female was referred to the Biochemical Diseases Clinic at BC Children’s Hospital. She was from a non-consanguinous Caucasian white family from New-Zealand on maternal side and Canada on paternal side. Both sides’ ancestries came from the British Isles. She started having symptoms after the age of 10 years, and was followed in the Pediatric Neurology Clinic. She was from a sibship of four, she had a healthy older sister, and an older sister who had migraines and seizures, and a younger brother with migraines. Family history revealed a maternal grandfather with history of debilitating migraines, who died of a brain tumour, and a mother with history of migraines.

2.1.1 Family history

Clinical information on a four generation pedigree suggests an autosomal dominant inheritance with variable expressivity and with gender difference (Figure 12).

![Family pedigree](image)

Figure 12. Family pedigree
2.2 Clinical phenotype

2.2.1 Proband (III-3)

She is the third child born to this couple. Pregnancy and delivery were unremarkable. Early developmental milestones were within normal limits. Since the age of 11 years she started having episodes of hemiplegic migraines. These presented with hemiparesis, aphasia followed by severe headaches, nausea, photophobia and phonophobia. The hemiparesis and numbness alternated between the two sides. Initially these episodes occurred every 3-8 weeks, the acute phase lasting between 4-48 hours, but the numbness and headache lasting sometimes up to 7 days, followed by full recovery. The frequency and severity of the migraine episodes increased over the years. They occurred twice a month at the age of 16 years. Her migraines were resistant to standard migraine therapy.

From the age of 11 years she had progressive deterioration in her gait with ataxia and progressive spastic paraparesis, at age 14 requiring crutches to walk, and at 17 using mostly a wheelchair. At 17 years she started treatment with a combination of 5-hydroxytryptophan (5HTP) and carbidopa, and her lower limb strength improved to a point that she again walked with crutches and no longer was wheelchair-bound. This treatment was commenced because she was found to have low cerebrospinal fluid (CSF) levels of serotonin metabolite, 5-hydroxyindoleacetic acid (5HIAA).

At the age of 13 years she developed sensory loss in stocking distribution in her lower limbs. At age 17 sensation in her lower limbs was so impaired that she had second degree burns on her feet from a hot water bottle that she couldn’t feel.

At this age neurological exam showed full extraocular movements, but there was some loss of fluidity and jerky saccades. Her pupils were equally reactive to light and funduscopy was normal. She had paucity of facial expression but no facial asymmetry. Her gag was impaired. She was able to stand and walk a few steps without her crutches but her balance was poor and she had positive bilateral Trendelenburg gait. She had mild contractures of her elbows but normal power in her upper limbs. She had weakness in her lower extremities most marked at her hip flexors (3/5). Her plantar responses were extensor. She had mild contractures in her knees, and increased tone in her lower limbs. Deep tendon reflexes were brisk. Light touch sensation
was decreased in her lower extremities up to her knees and vibration sense was impaired. Her position sense was intact. She had mild scoliosis.

She had enuresis since age 7 years after a severe viral respiratory illness, and she developed other urinary symptoms by age 16, such as urge incontinence, frequency, and stranguria (painful urge to urinate). She also had repeated episodes of cystitis, treated with antibiotics. Urodynamics study showed a large capacity, insensate, atonic bladder with evidence of increased post-void residual. In recent years she had to catheterize herself every two hours, as she had lost control of her voiding.

Her bowel movements initially were characterized mostly by constipation, but later she developed a diarrhea predominant irritable bowel disease, which she treated with diet and loperamide.

She had mild dysarthria and dysphagia, sleep problems, anxiety, and intermittently depressed mood. She also had frequent episodes of orthostatic dizziness and she fainted on the tilt table test, when she was upright.

At the age of 15 years she had a large (measuring 6.5 cm x 1.2 cm x 1.2 cm) right ovarian tumour removed, diagnosed on pathology as benign serous cystadenofibroma. At age 19 she had multiple breast tumours removed. The final pathology diagnosis was: left breast fibroadenoma and right breast benign phylloides tumour. This could be relevant considering the history of the maternal grandfather’s brain tumour.

She was cognitively intact and she started university aspiring to become a graphic artist, as she was an excellent cartoonist. At the age of 21 years, soon after starting her studies at university, she had a severe episode of migraine that lead into a prolonged episode of coma. Prior to this episode her migraine headaches were mild and less frequent, the last migraine prior to this was 2 months before, and there was no history of head trauma or any other triggers for this severe episode. On admission to the local hospital she had fever in the range of 38 – 39°C, tachycardia of 140 bpm, dilated pupils and moderate generalized rigidity, and she was poorly responsive. Toxicology studies were negative. She required intubation and admission to the intensive care unit. She developed unusual increased muscle tone associated with fluctuating bifacial spasms that resembled seizures. EEG recordings did not suggest epileptic activity. She was treated with supportive care and gradually she improved. After 2 months she was transferred to the step down unit. She required a feeding tube and tracheostomy. She was poorly responsive for about ten weeks, and then she gradually started to show signs of writing and
nonverbal communication. She might have suffered hypoxic ischemic brain injury during the coma. When she started talking, she was severely dysarthric, and this never improved. She communicated mostly with a letter board. She was transferred to the rehabilitation centre where she remained for another three months. Upon discharge, she was cognitively intact, had severe dysarthria, but no specific cranial nerve findings. She had normal strength in her upper extremities but severe weakness in the lower extremities with no antigravity hip flexion or ankle dorsiflexion. Vibration threshold was reduced at the toes. Sensation to temperature was severely diminished in a stocking distribution. She was self-catheterizing, on a bowel protocol, transferring with a sliding board or with a standing pivot transfer with a one-person heavy assist.

She was not given treatment with 5HTP/carbidopa during her ICU stay, or recovery.

Patient developed seizures 10 days after discharge from the rehabilitation unit. First she had a generalized tonic-clonic seizure then she developed left face twitching. These were difficult to control, and she was admitted several times to hospital. She was managed with valproic acid, topiramate, phenytoin and levetiracetam.

She was transferred for rehabilitation to the Acquired Brain Injury Ward, and her Montreal Cognitive Assessment (MOCA) score at this time was 17/30. This was the first time that cognitive deficit was documented in her. She was a cartoonist and she had difficulties drawing a clock.

Her seizures remained intractable, and she was discharged home to a palliative care situation. She lost all communication capacities, was aphasic and confused. She could no longer recognize letters to form words on the communication board. She died in her hometown 15 months after the episode with coma.

### 2.2.2 Proband’s sister (III-2)

She’s the product of normal pregnancy and delivery. Early development was normal. She started developing symptoms after a serious viral illness at around the age of 11 years. She began to have difficulties walking upstairs, and this has become worse over time. She had to quit her sports teams in grade 9 and 10 because of leg pain and weakness.

She started getting headaches around the age of 11. They would start with numbness and tingling in her mouth, and weakness on one side affecting her arm and leg. The headache would last for about one day, the weakness for 1 to 2 days. It would be made better by sleep. Her first
A neurometabolic clinic visit was when she was 19 years old. Neurological exam revealed a slim girl with poor muscle bulk. Her tone was slightly increased in her lower limbs, and power showed significant proximal weakness with evidence of fatigability. Gower’s maneuver was positive. Deep tendon reflexes were 2+ and symmetrical in both upper and lower limbs. Her plantars were down going. Abdominal reflexes were absent. Sensory examination appeared to be normal. She did have resting tremor of her hands. Her gait was stiff and she had difficulties running, and she fell during gait examination. She had difficulties with Tandem gait.

She had problems with mood swings, particularly depression. She did have some bladder accidents at night or when she was tired, and she had urgency.

She had twitching and myoclonus, but she never had a generalized tonic clonic seizure. EEG was epileptiform and she started treatment with Valproic acid.

Cognitively she was unaffected, she was attending university and was getting A’s and B’s. Later on she graduated with a Bachelor degree in economics.

Over the following years she had a slow deterioration of her gait, and power, showing more significant proximal weakness. This has improved with 5HTP/carbidopa supplementation. This treatment has been also commenced because she had low levels of CSF serotonin metabolite 5HIAA.

At the age of 24 she became pregnant and her clinical condition deteriorated significantly during pregnancy. We discontinued the valproic acid and 5HTP/carbidopa for about a month in her first trimester but she had three severe migraine episodes associated with delirium and the decision was made to restart the valproate and for her strength the 5HTP. She started taking a larger amount of the 5HTP/carbidopa in her second trimester because she did not have the strength to walk without it. Extensive literature review on this medication’s effects on the pregnancy have not come up with any teratogenic effects on the fetus, but we did find evidence that low serotonin is detrimental to fetal development. We tried to limit her 5HTP intake to maximum four times a day in her last trimester to avoid the remote possibility of serotonin withdrawal in the newborn. She became wheelchair-bound in her last two months of pregnancy. A team of medical internist, feto-maternal specialist and obstetrician followed her closely during pregnancy. She had several detailed fetal ultrasounds, which were normal.

Her delivery was a planned C-section at 36 weeks, and she was admitted to the hospital two weeks prior to the date of the delivery. Anaesthesia was done with epidural infusion. She tolerated the procedure well, although she became very tired and had low blood pressure by the
end. She had tubal ligation after the delivery of the newborn. A healthy newborn baby girl was delivered with a birthweight of 2380 g. Apgars were 91 and 95. She was admitted to the Neonatal Intensive Care Unit for observation. She had nasogastric feeds with formula for the first 2 weeks of life, then she transitioned to bottle feeds. The decision not to breastfeed was made because we had no safety information on breastfeeding and taking 5HTP/carbidopa. Our patient was observed for 24 hours in the Intensive Care Unit postpartum, and then she was discharged to a regular ward. Initially she was extremely fatigued and weak, but slowly she was regaining her strength with the 5HTP/carbidopa dose increase. She had no migraine episodes. Mother and newborn were discharged home in good clinical condition after 2 weeks in hospital. There were no signs of post-partum depression. The infant is developing well, and currently she is a healthy 2-year-old toddler.

Our patient was found to have slow clinical deterioration over the next 2 years. She had lost weight and became very frail. Her lower limb strength worsened, and she tried to take more 5HTP with carbidopa, unfortunately Health Canada denied further Special Access to carbidopa even though it is FDA approved in the USA. She tried to take only 5HTP but she had developed severe diarrhea, then we tried to combine it with L-dopa, but she still had GI side effects. She continues to feel the immediate benefits in her strength when she takes the combination of 5HTP/carbidopa that she keeps for special occasions.

An N=1 trial proposal has been requested from Health Canada for proving efficacy and safety of carbidopa in combination with 5HTP (Appendix A, B), awaiting approval.

There are many similarities between the clinical course of the two sisters, being both severely affected, but the younger sister had a more complicated course because of the coma.

At age 27 years, she became depressed and irritable and she needed a short admission to a Neuropsychiatric Unit. During the admission her neurological exam documented well the difference between tone and strength in her lower limbs before and after administration of the 5HTP/carbidopa combination. Prior to the medication her tone in the lower extremities was increased, with 4/5 strength at the hips. After the 5HTP, her tone was normal with iliopsoas strength of 5/5.

At her most recent clinic visit, she was found to have significant difficulties walking, needing a cane. She lost more weight, with BMI of 17. Her appetite is poor and she doesn’t feel like eating most days. Her muscle bulk was diminished compared to previous and her strength was down to 4/5 in the upper proximal muscles and 2-3/5 at the hips. She had difficulties
standing up from a sitting position. Deep tendon reflexes were brisk with positive lower limb cross adductor response bilaterally. Sensation to touch and temperature were normal in the legs and feet. She had spastic gait and difficulties with Tandem gait, and she also had mild resting and intention tremor of both hands. She has dysarthria and perhaps a slight tremor of her voice. MOCA score was 24/30.

2.2.3 Proband’s brother (III-4)

He is the youngest sibling, born after an unremarkable pregnancy, by normal delivery and no neonatal complications. His early childhood development was age appropriate. He has never had problems with strength or exercise tolerance. He has been very slim all his life.

He had his first migraine in grade 6. This involved the left side of his face and tongue. The episode lasted for about 20 minutes, followed by a throbbing headache and vomiting. The headache lasted for about 24 hours. Since then he had an average of 4 similar episodes per year. These always affect the left side of his face and tongue and occasionally involve his right leg and then he cannot walk. He gets slightly confused during these episodes and cannot speak.

His first assessment in the metabolic clinic was at the age of 19 years. He complained of getting dizzy and lightheaded upon standing up, and jerking and twitching of his legs, with falls to the ground. His other symptoms were: chronic constipation and lately sexual dysfunction and bladder urgency. He was enrolled in a college BA program. The only positive finding on his neurological exam initially was brisk (+3) deep tendon reflexes overall, with two beats of clonus bilaterally. His BMI was 17.9.

Over the next year he experienced an increase in the frequency of his migraines, occurring every 3-4 month. An EEG showed epileptiform activity, and he was started on Valproic acid.

At his most recent clinic visit there was a noticeable decrease in his power at the hips to 4/5. He complained of anxiety but not of depressed mood.

2.2.4 Mother of the proband (II-2)

Has history of severe migraines as a young woman, but not recently. She does not complain of weakness, exercise intolerance of fatigue. Investigations were performed at the age of 50 years. Her father (I-2) had debilitating migraines throughout his life, and died in his 60’s of a brain tumour.
A summary of the clinical findings in the affected members of the family can be found in Table 1.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Family member</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>III-3</td>
</tr>
<tr>
<td>Hemiplegic migraines</td>
<td>+++</td>
</tr>
<tr>
<td>Spastic paraparesis</td>
<td>+++</td>
</tr>
<tr>
<td>Seizures</td>
<td>+++</td>
</tr>
<tr>
<td>Sensory loss</td>
<td>+++</td>
</tr>
<tr>
<td>Bladder instability</td>
<td>++</td>
</tr>
<tr>
<td>Dysarthria</td>
<td>++</td>
</tr>
<tr>
<td>Tumours</td>
<td>++</td>
</tr>
<tr>
<td>Coma</td>
<td>+</td>
</tr>
<tr>
<td>Response to 5HTP/ carbidopa</td>
<td>+++</td>
</tr>
</tbody>
</table>

Table 1. Summary of clinical symptoms of affected members of the family.

2.3 Treatment

Medications used for the neurotransmitter deficiency were 5-hydroxytryptophan (5HTP) administered together with carbidopa in a 4:1 ratio with maximum of 100 mg 5HTP + 25 mg carbidopa /dose was given for the serotonin deficiency. L-dopa/carbidopa 100/25 combination, 25 mg L-dopa TID was also added for the dopamine deficiency. Leucovorin 5 mg OD was added, to prevent cerebral folate deficiency, that can be associated with dopamine treatment.

The proband started taking 100 mg 5HTP/25 mg carbidopa TID, at the age of 17 years, and in about 6-8 months she was able to ambulate more freely, only using her crutches, and even independently for short distances, needing her wheelchair only for outside.

The proband’s sister had an even more dramatic response, especially during her pregnancy, she was taking 50 mg 5HTP/12.5 mg carbidopa and in half an hour she would have enough strength to walk short distances, shower, or carry out some errands. By the end of her second trimester she was taking this dose up to 9 times a day without any visible side effects. After the delivery she continued to have good response in her lower limb strength with the same dose taking 7-8 times a day.

We were not able to start treatment in the brother because of lack of special access approval for carbidopa from Health Canada.

We have written an emergency letter recommending 10 mg of intranasal ketamine if the migraine aura lasts more than 12 hours, to try to abort the CSD. This regime has been recommended after the proband had her coma episode, which never recurred and the sibling did
not yet have a prolonged episode of the aura, to warrant administration of ketamine. The emergency letter is still in effect for the two living affected siblings.

2.4 Investigations: MRI

2.4.1 Proband

Initial MRI showed T2 symmetrical hyperintensities in the pons and external capsules. The pons was also small. (Figure 13).

Figure 13. Initial MRI proband
(a) Symmetric T2 hyperintensities within the anterior belly of the pons; (b) High signal intensity within both external capsules; (c) Small pons; (d) Sagittal image of narrow spinal cord.
The sagittal T2 weighted sequence of the spine demonstrated a circumferentially small spinal cord involving its entire length (Fig 13d.). On axial images in the cervical cord there were symmetrical T2 hyperintense tracts, most evident at C3 and C4 levels.

MR spectroscopy showed a slightly elevated choline, a marker of cellular membrane turnover.

After the coma episode there were extensive areas of new abnormal signal in the cerebral cortex with associated volume loss of cortical encephalomalacia, involving the anterior temporal lobes bilaterally, the right frontal lobe just above the insula, the right parietal lobe more than left, and medial frontal lobes bilaterally. New diffusely abnormal signal was seen in the bitemporal gyri, bifrontal gyri, insular cortex bilaterally, cingulate gyrus bilaterally and frontoparietal gyri/subcortical white matter, without reduced diffusion. Gyral and subcortical white matter abnormality developed in the right posterior frontal and adjacent right parietal lobes, with a progression in gyral and subcortical white matter signal abnormality in the left parietal lobe (Figure 14). The time interval between the initial MRI and the one after the coma episode is 5 years.

**Figure 14.** Extensive white matter changes after coma episode
Arrows end at the affected white matter regions.
2.4.2 Proband’s affected sister

At age of 19 years there was mild cerebellar atrophy with slight enlargement of the fourth ventricle. Abnormal, symmetric, band-like high T2 signal intensities were identified anteriorly in the pons. Additionally, abnormal high signal intensity was also identified in the external/extreme capsules bilaterally. Subtle increased T2 high signal was identified around the occipital horn of the left lateral ventricle. There was also generalized atrophy of the spinal cord (Figure 15). MR spectroscopy showed mildly elevated choline.

![Figure 15. Proband’s sister’s initial MRI](image)
(a) T2 hyperintensity in pons (b) Symmetrical hyperintensities in external capsule (c) Small belly of the pons (d) Sagittal view of narrow spinal cord
Arrows end at the described affected areas.
2.4.3 Proband’s affected brother

At age 19 years: there were white matter lesions particularly involving the subcortical and juxtacortical white matter of the right anterior temporal lobe and the medial superior frontal lobes bilaterally. These white matter changes were suspicious of CADASIL according to the neuroradiologist’s report. There was mild volume loss involving the brainstem. There was also moderate volume loss throughout the spinal cord. Small areas of increased T2 weighted signal intensity were seen within the cervical spinal cord, extending from C2 to the cervicothoracic junction involving the most lateral aspects of the spinal cord bilaterally (Figure 16).

![Figure 16. MRI of proband’s brother](image)
(a) Small T2 hyperintensities in the frontal white matter (b,c) Sagittal images of narrow spinal cord

2.4.4 Proband’s mother

There were multiple T2 hyperintense lesions measuring up to 5 mm within the white matter of the left and right frontal lobes. These lesions are very similar to her son’s. The spinal cord was slightly narrow in the thoracic region, with small signal changes on T2 in the cervical region on lateral aspects (Figure 17).
Figure 17. MRI of proband’s mother
(a) Small T2 hyperintense areas in frontal white matter (b) Sagittal view of mildly narrow thoracic spinal cord (arrow ends at the affected regions)

2.5 Biochemical investigations

2.5.1 Proband

Metabolic screening investigations revealed: normal ammonia, lactate, plasma amino acids, acylcarnitine profile, folate, vitamin B12 levels, CPK, acetylcholine receptor antibodies, double-stranded DNA, anti-nuclear antibody (ANA), copper, ceruloplasmin, arylsulfatase-A, serum transferrin isoelectric focusing, urine organic acids, purines & pyrimidines and urine creatine, CSF glucose, lactate, protein, amino acids, oligoclonal bands.

CSF neurotransmitter results (performed by Medical Neurogenetics Laboratory, Atlanta, USA) (at age 17, 18 and 22) showed low levels of serotonin metabolite 5-hydroxy-indoleacetic acid (5HIAA), normal homovanillic acid (HVA), tryptophan, and biopterin metabolites (neopterin and tetrahydrobiopterin) (Table 2).
Table 2. CSF neurotransmitter levels in proband at ages 17, 18 and 22 years

Platelet serotonin levels were measured at the Health Diagnostics and Research Institute, formerly Vitamin Diagnostics (in South Amboy, NJ, USA) at baseline, and after starting treatment with 5HTP/carbidopa (Table 3). The dose of the 5-hydroxytryptophan was increased after the first 6 months from 50 mg TID to 75 mg TID, and after the first year to 100 mg TID. The 5-hydroxytryptophan/carbidopa ratios were kept the same, at 1:4.

Table 3. Serial platelet serotonin measurements in proband

<table>
<thead>
<tr>
<th>Time of measurement</th>
<th>Platelet serotonin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ref: 65-550 ng/10E10</td>
</tr>
<tr>
<td>Baseline</td>
<td>18.3</td>
</tr>
<tr>
<td>6 months on 5HTP/carbidopa</td>
<td>39.4</td>
</tr>
<tr>
<td>1 year on 5HTP/carbidopa</td>
<td>48.6</td>
</tr>
<tr>
<td>2 years on 5HTP/carbidopa</td>
<td>80.6</td>
</tr>
</tbody>
</table>

2.5.2 Proband’s affected sister:

CSF neurotransmitter results (performed by Medical Neurogenetics Laboratory, Atlanta, USA) showed low levels of serotonin and dopamine metabolites and normal tryptophan (Table 4).

Table 4. Proband’s sister’s CSF neurotransmitter levels at baseline

<table>
<thead>
<tr>
<th>5HIAA ref: 67-189 nmol/L</th>
<th>HVA Ref:167-563 nmol/L</th>
<th>Tryptophan Ref: 1.26-3.8 µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>105</td>
<td>2.66</td>
</tr>
</tbody>
</table>

Platelet serotonin levels were measured at Health Diagnostics and Research Institute, at baseline and several times after starting the 5HTP/carbidopa treatment (Table 5). 5HTP dose was gradually increased during second trimester in pregnancy, lowered in the third trimester, and doubled after delivery.
<table>
<thead>
<tr>
<th>Time of measurement</th>
<th>Platelet serotonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>27.1</td>
</tr>
<tr>
<td>1 year on 5HTP/carbidopa</td>
<td>36.9</td>
</tr>
<tr>
<td>2 years on 5HTP/carbidopa</td>
<td>80.7</td>
</tr>
<tr>
<td>2\textsuperscript{nd} trimester in pregnancy</td>
<td>62.3</td>
</tr>
<tr>
<td>3\textsuperscript{rd} trimester in pregnancy</td>
<td>40.6</td>
</tr>
<tr>
<td>4 years on 5HTP/carbidopa (2 years after delivery)</td>
<td>161.3</td>
</tr>
</tbody>
</table>

Table 5. Serial measurements of platelet serotonin levels in proband’s sister, at baseline and on treatment with 5HTP/carbidopa

2.5.3 Proband’s brother

CSF neurotransmitter results (performed by Medical Neurogenetics Laboratory, Atlanta, USA) revealed very low level of serotonin metabolite (5HIAA) and low level of dopamine metabolite (HVA) (Table 6).

<table>
<thead>
<tr>
<th>5HIAA</th>
<th>HVA</th>
<th>Neopterin</th>
<th>Tetrahydrobiopterin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ref: 67-189 nmol/L</td>
<td>Ref: 167-563 nmol/L</td>
<td>Ref: 8-28 nmol/L</td>
<td>Ref: 10-30 nmol/L</td>
</tr>
<tr>
<td>18</td>
<td>99</td>
<td>12</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 6. Proband’s brother CSF neurotransmitters at baseline

Platelet serotonin levels were measured at baseline at the Health Diagnostics and Research Institute and were below the reference range (Table 7).

<table>
<thead>
<tr>
<th>Time of measurement</th>
<th>Platelet serotonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>40.7</td>
</tr>
</tbody>
</table>

Table 7. Proband’s brother platelet serotonin levels at baseline

2.5.4 Proband’s mother:

CSF neurotransmitter results (performed by Medical Neurogenetics Laboratory, Atlanta, USA) showed the level of the serotonin metabolite (5HIAA) close to the lower end of reference range and normal dopamine metabolite (HVA) (Table 8). The low normal 5HIAA level could be in keeping with the mother’s milder clinical phenotype.
### Table 8. Proband’s mother’s CSF neurotransmitters at baseline

<table>
<thead>
<tr>
<th>5HIAA</th>
<th>HVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ref: 67-189 nmol/L</td>
<td>Ref: 167-563 nmol/L</td>
</tr>
<tr>
<td>69</td>
<td>204</td>
</tr>
</tbody>
</table>

Platelet serotonin levels were also measured at Health Diagnostics and Research Institute and were borderline low (Table 9).

<table>
<thead>
<tr>
<th>Time of measurement</th>
<th>Platelet serotonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Ref: 65-550 ng/10E10</td>
</tr>
<tr>
<td></td>
<td>51.2</td>
</tr>
</tbody>
</table>

### Table 9. Proband’s mother’s platelet serotonin levels at baseline

#### 2.6 Other investigations

##### 2.6.1 EEG, EMG, NCS, bone mineral density, and molecular investigations

**EEG** in the proband initially showed mild dysrhythmic background, but no epileptiform activity initially. After the coma episode there was mild to moderate slowing and dysrhythmia on the right side. On the left, there was moderate slowing. This is suggestive of a mild to moderate encephalopathy. In addition seizures were also recorded and a build-up of epileptiform activity was seen over the left hemisphere with prominent involvement of the left fronto-parietal region.

**Other electrophysiological studies** were performed in the proband, and visual evoked potentials were normal.

**EMG & Nerve conduction studies** were performed in the proband at age 16 and 20, and they remained normal. There were no findings of generalized neuropathy or peripheral entrapment neuropathy identified in the upper or lower limbs. There were no findings of a myopathic process or anterior horn cell process identified in the study.

**Bone mineral density** was also performed in the proband at age 20, and showed an average T-value of lumbar spine of 1.6 standard deviation below mean, consistent with
osteoporosis, and an average T-value of left hip of 3.2 standard deviation below mean, also consistent with osteoporosis.

**Molecular investigations** (clinical testing done at Medical Neurogenetics Lab in Atlanta, USA) that were done in the proband and were negative for the following individual genes: myotonic dystrophy (**DMPK**), FHM-related genes (**CACNA1A**, **ATP1A2**), mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes (MELAS) common mutations.

### 2.6.2 Biopsies

**Muscle biopsy** (performed in the proband at BC Children’s Hospital and interpreted by Pathology and Biochemical Genetics Laboratories) revealed a normal microscopy, histochemistry staining revealed fibre type 1 predominance, and electron microscopy showed normal mitochondria. Mitochondrial respiratory chain enzyme assays revealed normal Complex I, II, IV activities and ratios to the marker enzyme, citrate synthase, were also normal. Mitochondrial DNA extracted from muscle was checked for the common MELAS, MERRF and NARP mutations. Mitochondrial inheritance could be considered if we assume that the grandfather is not affected, but not supported by the lack of elevated lactate in blood and CSF during the end stage disease of the proband, the normal muscle biopsy, and the selected molecular investigations.

**Full thickness rectal biopsy** was performed in the proband at age 18 to look for intranuclear inclusions, showed few ganglion cells in the myenteric plexus. There were some bands of Bungner and some collagen pockets present suggesting that there is axonal loss and that there is autonomic involvement of the disease. There were no intranuclear inclusions seen.

There were no peripheral nerve or intestinal biopsies performed.

### 2.6.3 Functional studies

**Tilt table testing** (performed at BC Children’s Hospital and interpreted by myself) revealed in the proband a baseline heart rate of 60 bpm, blood pressure (BP) of 105/62 mmHg. Head-up tilting (HUT) at 70° resulted in increase in heart rate to 106 bpm, and BP to 120/70 mmHg with symptoms of dizziness, lightheadedness, nausea, sweating, and a syncopal episode at 7 minutes upright, with drop in BP and HR. She recovered to baseline HR and BP after 5
minutes supine (Figure 18). These results are suggestive of Postural Orthostatic Tachycardia syndrome, and thus cardiac autonomic dysfunction.

![HR response to HUT](image1.png) ![BP response to HUT](image2.png)

**Figure 18.** Tilt table testing results in the proband (a) heart rate and (b) blood pressure response to head-up-tilt (↑arrow head-up-tilt, ↓arrow supine)

Plasma catecholamines were drawn at the end of 20 minute supine and at the end of the upright phase from an indwelling venous catheter and showed inadequate plasma norepinephrine response to the head-up tilt (Table 10).

<table>
<thead>
<tr>
<th>Position</th>
<th>Plasma epinephrine</th>
<th>Plasma norepinephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supine</td>
<td>0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>Upright</td>
<td>0.77</td>
<td>0.36</td>
</tr>
</tbody>
</table>

**Table 10.** Plasma catecholamine levels during the tilt table test’s supine and end of upright phase

**Hearing test** performed in the proband at age 21 years showed a mild high frequency sensorineural hearing loss at 6-8kHz on the left side, and at 4-8kHz on the right side.

**Lung function test** was performed in the sister of the proband as she had shortness of breath on exercise, and showed mildly reduced vital capacity. The FEV1/FVC ratio was normal. The diffusion capacity was mildly reduced (Table 11).

<table>
<thead>
<tr>
<th>Spirometry</th>
<th>Lung volumes</th>
<th>Diffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVC 74%</td>
<td>TLC 87%</td>
<td>DLCO 74%</td>
</tr>
<tr>
<td>FEV1 80%</td>
<td>VC 76%</td>
<td>KCO 91%</td>
</tr>
<tr>
<td>FEF25-75 104%</td>
<td>IC 72%</td>
<td></td>
</tr>
</tbody>
</table>

**Table 11.** Lung function tests in the sister of proband
- FVC – forced vital capacity, FEV1- forced expiratory volume in 1 second, FEF25-75- forced expiratory flow 25-50%, TLC – total lung capacity, VC- vital capacity, IC – inspiratory capacity, DLCO – diffusing capacity, KCO- transfer coefficient
2.6.4 Post-mortem brain histopathology

Post-mortem brain histopathology was performed by Dr. Wayne Moore, Neuropathologist at Vancouver General Hospital. On the axial horizontal sections of the brain there are small areas of cystic encephalomalacia (softening or degeneration of brain tissue) involving both posterior frontal and anterior parietal lobes, involving mainly the white matter and, to a lesser extent, deep cortical grey matter (Illustration 1). This could be indicating a previous cerebral hypoxic insult, or an underlying leuco-encephalopathy.

Illustration 1. Axial sections of brain of the deceased proband
Photographs of axial sections of the brain obtained at the autopsy of the proband show areas of encephalomalacia at the end of the arrows.

2.6.5 Microscopic description

In the medulla there was hypercellularity, predominantly oligodendroglial, in the descending tracts of the basis pontis. There was minimal perivascular chronic inflammatory infiltrate in the midline in the medulla.

In the midbrain there was mild hypercellularity of the tectum. Cerebral peduncle was normal.

In the frontal subcortical area there was cavitating encephalomalacia within the white matter and to some degree the deeper cortex with involvement of the subcortical U-fibers. Numerous lipid-laden macrophages were seen in this area. There were rare vessels with hyalinized walls seen in the margin of the lesion. Many small round vessels, consistent with oligodendrocytes were seen in the lesional wall. On one area in the subcortical U-fibre region
the appearance was vacuolar. Occasional atypical nuclei were seen in the cortex. There was hypercellularity, in which there were numerous positive CD68-positive cells (probably macrophages) but none of the small round cells were CD45 (leukocyte common antigen)-positive, suggesting they are oligodendrogial, many of them being myelin basic protein (MBP) positive. The Prussian blue stain showed only very rare cells containing stainable iron. Staining for beta-amyloid protein (beta-App) showed accumulation of the stain, indicating axonal damage, usually seen post-hypoxia, or trauma, but also in acute multiple sclerosis, amongst others.

The overlying cortex showed increased numbers of blood vessels with prominent endothelium (neovascularization) that could be seen amidst relatively normal appearing neurons.

Axons appeared to retain their myelin sheaths in these regions, as evident on the LFB-Bielshovsky stain and the immunohistochemistry stain for MBP. Immunohistochemistry showed aggregates of synaptophysin in axons in the white matter, some of which appeared to be in distended axons. There were scattered cells positive for p53 in the cortex, including atypical cell, as well as cells in the white matter (differentiate malignant cells). The atypical cells were positive for glial fibrillary acidic protein (GFAP) indicating they are glial cells, and negative for CD34 (marker of hematopoietic progenitor cells and endothelial cells), synaptophysin and phosphorylated neurofilament. There were occasional Ki67-positive cells (staining neurofilament) in the cortex and white matter, but most of the atypical cells were Ki67-negative.

The descending tracts of the basis pontis showed vacuolar change. This spared the crossing pontocerebellar fibres and the middle cerebellar peduncle. The pontine tegmentum was normal. There was encephalomalacia within the inferior temporal and occipitotemporal white matter. This tended to involve the superficial gyral white matter where U-fibres are involved and there are macrophages. Deeper there was hypercellularity in the gyral white matter and still further deeper vacuolar change is noted with a lesser degree of hypercellularity. The Bielschovsky stain showed no abnormalities in the gray matter and axons (while reduced in number) running through the encephalomalacic white matter. Immunohistochemistry for Tau was negative. Immunohistochemistry for ubiquitin, TDP43 and alpha-synuclein were within normal limits (these stains are ALS specific).

There was encephalomalacia restricted to the extreme capsule, associated with hypercellularity of the white matter of the insula. The insular cortex showed occasional atypical
nuclei. The putamen, internal capsule, and globus pallidus were normal. The external capsule showed hypercellularity.

There was mild vacuolar change and rarefaction in the white matter of the anterior and posterior vermis with focal hypercellularity in the area of vacuolation. There were characterized by clusters of small round cells consistent with oligodendrocytes (Figure 19 and Figure 20).

**Figure 19.** Histopathology of brain slides obtained from autopsy from the deceased proband
Bizarre cortical glial cells stained with (a,b) H&E and (c) GFAP, (d) Leukomalacia low power (e) Leukomalacia 10x
Figure 20. Histopathology of brain slides obtained from autopsy from the deceased proband
(a) Beta-App accumulation (b) Vacuolation of white matter (c) Synaptophysin white matter (d) Hypercellular white matter

The final comment from the neuropathologist was that the focal encephalomalacia associated with hypercellularity and vacuolation in several areas can be related to ischemia or to a vacuolar leukoencephalopathy. Considering that the vacuolation was only in the deeper regions of the gyral matter, one hypothesis is that the changes are manifestations of an axon transport disorder affecting the distal aspects of ascending projections to the cortex. The finding of aggregates of synaptophysin immunoreactivity in axons in the white matter is also consistent with a disturbance of axonal transport. There was an oligodendroglial response to the axonal abnormalities. There were also atypical astrocytes noted in the cortex. Their expression of p53 would favour their being neoplastic. They were, however, only scattered and in the absence of an infiltrating pattern this cannot be regarded as glioma per se. However, their presence is of
considerable interest in as much as the proband manifested a propensity to neoplasia, and the maternal grandfather had a brain tumour.

There was no full autopsy performed as per the family’s wishes, only the brain was removed postmortem.
Chapter 3: Hypothesis driven research

3.1 Generating a differential diagnosis and an etiological hypothesis based on clinical and biochemical phenotype

3.1.1 Differential diagnosis based on neuroanatomy and neuroradiology

Based on the clinical symptoms of progressive lower limb spasticity, brisk deep tendon reflexes and weakness, an upper motor neuron (UMN) descending tract lesion is high on the list of the differential in this family. The bilateral leg weakness with the flexors being more affected than extensors, is consistent with a pyramidal pattern (pyramidal tracts). There are subtle signs of brain stem involvement with the dysarthria, dysphagia, and spinothalamic sensory loss. Lesions in the pons above the pyramidal decussation laterally involve the spinothalamic tracts, causing loss of pain and temperature sensation. Lesions at the base of the pons (middle and inferior cerebellar peduncle) cause ipsilateral limb and gait ataxia. Preservation of proprioception and vibration sense, but loss of pain and temperature sensation in the lower limbs point towards the involvement of the anterior lateral and lateral columns (spinthalamic) in the spinal cord.

The descending corticospinal tracts are involved only starting in the pons, as there is no evidence of internal capsule involvement on brain MRI. The external capsule hyperintensities on the MRI suggest involvement of commissural and associative fibers and inferomedial thalamic peduncle. Fibers arise inferiorly and medially and ascend vertically.

The spinal cord atrophy was unchanged over the years, pointing towards a developmental, rather than degenerative cause.

Based on the UMN involvement, Amyotrophic Lateral Sclerosis (ALS) and Spinal Muscular Atrophy (SMA) are the two most common motor neuron diseases that are in the differential diagnosis. Hereditary spastic paraparesis (HSP) and Spinocerebellar ataxias (SCA’s) are also in the differential diagnosis, considering the spinal cord and cerebellar involvement. CADASIL was also raised in the differential diagnosis on the MRI changes in the brother and the mother.

In the family investigated here there were many similarities in either clinical or biochemical symptoms with the above named neurodegenerative disorders, but none of them
with perfect fit. Symptoms were suggestive in some aspects but not others, of HSP, SCA, ALS, MS, CADASIL, SMA, PD, FTD and MELAS. None of these disorders have associated classical hemiplegic migraines in their clinical presentation.

The proband had a normal muscle biopsy with normal muscle histochemistry and electron microscopy, and normal mitochondrial respiratory chain complex enzyme analysis. Mutations in the mitochondrial DNA associated with the MELAS phenotype were also ruled out. Mitochondrial DNA mutations were tested in muscle tissue.

### 3.1.2 Differential diagnosis based on migraine phenotype

The presentation with hemiplegic migraines was suggestive of FHM1, 2 or 3. In the pursuit of finding the etiology of the hemiplegic migraines we sequenced the *CACNA1A* and *ATP1A2* genes in the proband and both genes were negative for mutations. No cases with low CSF or platelet serotonin have been reported in either of these FHM syndromes.

### 3.1.3 Differential diagnosis based on biochemical phenotype

The low cerebrospinal fluid serotonin together with the low peripheral serotonin level directs toward a systemic serotonin deficiency. However since there have been no isolated tryptophan hydroxylase deficiency patients described in the literature, *TPH2* deficiency was hypothesized and the gene sequenced. Another cause for systemic serotonin deficiency could be serotonin transporter deficiency, also hypothetical, as no patients with SERT deficiency have been described before. Another possibility yet would be a gene defect in the serotonergic neuronal developmental pathway, such as *SPT5* or *FEV* gene defects.

These hypotheses were pursued with molecular and further biochemical investigations.

### 3.2 TPH2

#### 3.2.1 Introduction

Finding isolated significantly low CSF serotonin level in the proband lead to the hypothesis of primary tryptophan hydroxylase 2 (*TPH2*) enzyme deficiency. The *TPH2* gene was sequenced (chromosome 12: 93,595 base pairs, 490 amino acids, 11 exons).
3.2.2 Methods

Genomic DNA was isolated from blood with DNA Extraction Kit (Qiagen) and all exons and adjacent regions of the *TPH2* gene were amplified by standard PCR with Taq DNA polymerase and dNTP mix from Invitrogen. The oligonucleotide primers used were ordered from NAPS Unit, a core facility at Michael Smith Laboratories at UBC. Primers are detailed in Table 12 and Table 13.

#### Table 12. TPH2 Forward primers flanking all 11 exons.
Universal primer is highlighted.

<table>
<thead>
<tr>
<th></th>
<th>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</td>
</tr>
<tr>
<td>3</td>
<td>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</td>
</tr>
<tr>
<td>4</td>
<td>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</td>
</tr>
<tr>
<td>5</td>
<td>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</td>
</tr>
<tr>
<td>6</td>
<td>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</td>
</tr>
<tr>
<td>7</td>
<td>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</td>
</tr>
<tr>
<td>8</td>
<td>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</td>
</tr>
<tr>
<td>9</td>
<td>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</td>
</tr>
<tr>
<td>10</td>
<td>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</td>
</tr>
<tr>
<td>11</td>
<td>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</td>
</tr>
<tr>
<td>12</td>
<td>5′-TGTAAAACGACGGCCAGTCCCTTTATGTATTGTTCTCCACCCACCC-3′</td>
</tr>
</tbody>
</table>

#### Table 13. TPH2 Reverse primers flanking all exons.
Universal primer is highlighted.

<table>
<thead>
<tr>
<th></th>
<th>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</td>
</tr>
<tr>
<td>3</td>
<td>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</td>
</tr>
<tr>
<td>4</td>
<td>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</td>
</tr>
<tr>
<td>5</td>
<td>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</td>
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<tr>
<td>6</td>
<td>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</td>
</tr>
<tr>
<td>7</td>
<td>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</td>
</tr>
<tr>
<td>8</td>
<td>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</td>
</tr>
<tr>
<td>9</td>
<td>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</td>
</tr>
<tr>
<td>10</td>
<td>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</td>
</tr>
<tr>
<td>11</td>
<td>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</td>
</tr>
<tr>
<td>12</td>
<td>5′-CAGGAAAAACGCTATGACCGCGACAGAGGAAAGGAGG-3′</td>
</tr>
</tbody>
</table>

Amplification was carried out using an initial denaturation of 940 for 2 minutes, 600 or 650 or 680 for 90 minutes, 720 for 2 minutes, 29 cycles from step 1, and a final elongation of 720 for 8 minutes. All PCR products were purified using QIAquick PCR Purification Kit Protocol with microcentrifuge (QIAGEN) and visualized by running 5 µL of product on 6% polyacrylamide
gels. Purified products then were sent directly to the DNA Sequencing Core Facility at the Center for Molecular Medicine and Therapeutics (Vancouver).

3.2.3 Results

Sequencing revealed only common SNP’s in the *TPH2* gene.

3.3 Platelet serotonin uptake

3.3.1 Introduction

Serotonin transporter defect could lead to systemic serotonin deficiency as the transporter is the same in the CNS as it is in the periphery. Considering that SERT deficiency was the most likely diagnosis in our differential, based on the biochemical phenotype, platelet serotonin uptake experiments were designed and conducted.

3.3.2 Method

The experiments were performed as described previously\textsuperscript{155,156}. Blood collected in Na citrate tubes were used to get a platelet count, then submitted to a soft spin at 140 g x 10 min. All experiments were conducted in clear plastic tubes and at room temperature. The supernatant was removed to obtain platelet rich plasma (PRP), and the original tubes were subject to a hard spin at 2000 g. The supernatant was taken off, to obtain platelet free plasma (PFP). The PRP and PFP were mixed to obtain 200,000/µL platelet count, according to the following calculations:

\[
\frac{\text{plt #}}{200000} = 1 \text{ ml PRP} + x \text{ ml PFP}
\]

Labeled serotonin was prepared with \(^3\text{H}-\text{5HT}\) (Hydroxytryptamine creatinine sulfate 5-
\([1,2-\text{H}(N)], 5-\text{mCi}, 185 \text{ MBq}) [1 \text{ mCi (37 mBq) : 28.1 Ci/mmol (1.0397 TBq/mmol) Perkin-Elmer- NET498005MC}] in Krebs-Henseleit bicarbonate buffer \([(1.5 \mu l \text{ } ^3\text{H-5HT} + 200 \mu l \text{ buffer}},
\text{divided in 5 = 40} \mu l/\text{vial} \text{ to make up 9 vials of 40} \mu l \text{ (2.7} \mu l + 360 \mu l \text{ buffer}). Serial dilutions of \(^3\text{H-5HT}\) were made to obtain concentrations of \(10^{-8}, 2 \times 10^{-8}, 4 \times 10^{-8}, 6 \times 10^{-8}, 8 \times 10^{-8}, 10^{-7} \text{ and } 10^{-6} \text{ (µM)}.\]
Unlabeled serotonin (5HT Serotonin HCl Sigma H9523, FW 212.68) was prepared in serial dilutions of 1/10, 1/100 and 1/1000 (µM). First dilution was in buffer, then in HCl followed by serial dilutions in ddH2O (µM) (2 x unlabeled 5HT = 4.05 mg/5 ml buffer; then diluted in 20 µl HCl 0.1M).

Platelets were pelleted then diluted with pre-warmed buffer/serotonin at 37°C and incubated for 5 minutes, then the platelet activation was stopped by placing the vials on ice. After this the vials were centrifuged at 16,000 g for 10 min at 4°C and the supernatant was taken off. The pellets were washed with ice cold normal saline and centrifuged again 16,000 g for 10 min at 4°C. After the supernatant was taken off 100 µl SDS 0.1% was added and let stand for 30 min. 10 µl of the volume was taken out in 5 ml scintillation liquid in scintillation vials for counting. For total serotonin count the buffer/serotonin/platelet mix was used right after incubation; taking out 10 µl liquid and placed in 5 ml scintillation liquid in scintillation vials. For the bound serotonin count, the washed pellets in SDS 0.1% were used. Serotonin uptake was calculated using the formula: (bound count)/(total count).

The same experiment was repeated with added SSRI (serotonin reuptake inhibitor) Sertraline hydrochloride (Sigma sc-6319) (Mol wt: 342.7 AMU (g/mol); 1M=342.7 g/l, Solubility: DMSO ~26 mg/ml = 0.076 M (2.6 mg/100 µl), 10⁻⁴ dilution = 342.7 x 10⁻⁴ g/l = 0.03 x 10⁻⁵ g/l = 0.3 mg/100 ml) Serial dilutions of sertraline were also done with added platelets. The experiment was done using 8 controls and it was repeated twice in the proband (1a and 1b in Figure 21).

Results showed mildly diminished uptake in the proband’s affected sister, but almost no uptake in the proband. Two separate experiments were run in the proband (1a and 1b). One experiment had shown platelet serotonin uptake almost identical to the experiments where sertraline was added, blocking the serotonin transporter’s activity (Figure 21).
Figure 21. Serotonin uptake in controls and patients. (1a, 1b) proband’s experiments, (2) proband’s sister\textsuperscript{157}.

3.3.3 Transporter $K_m$ and $V_{max}$

To define the transporter kinetics in the proband with the low SERT activity we calculated $K_m$ and $V_{max}$ to demonstrate the rate of SERT uptake. Formulas used for $K_m$ and $V_{max}$ calculations were derived from the Michaelis-Menten equation\textsuperscript{158}

$$V = V_{max} \frac{[S]}{[S] + K_m}$$

S = substrate concentration

$V$ = velocity of reaction or number of moles of product formed per second

$V_{max}$ = maximal rate when the catalytic sites on the enzyme are saturated with substrate

$K_m$ = substrate concentration at which reaction rate is half its maximal value (Michaelis constant)

Since the classical double reciprocal method for calculating Michaelis constant ($K_m$) and the maximal reaction velocity ($V_{max}$) is prone to large errors due to distortion of the error structure, a
computer aided non-linear regression method was used to predict Vmax based on catalysis measurements at four different substrate concentrations.

SERT Km and Vmax were calculated for all samples. Results show significant decrease of Vmax and V calculated at a mean concentration level in the proband. Km was not significantly different. The proband had two separate experiments indicated in the rows Pt1a and Pt1b. The affected sister of the proband Pt2 had no difference in SERT kinetics, although her serotonin uptake in platelets was also lower than the -1SD below the mean of control samples’ uptake. (Table 14). The table shows excellent R2 correlation between measured and calculated values.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Km (10^{-7} µM)</th>
<th>Vmax (pmol/5min/2x10^5 platelets)</th>
<th>V at 15x10^{-7} µM</th>
<th>R2 (correlation with Michaelis-Menten equation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl1</td>
<td>11.1</td>
<td>5.37</td>
<td>3.1</td>
<td>0.9911</td>
</tr>
<tr>
<td>Ctrl2</td>
<td>7.5</td>
<td>4.89</td>
<td>3.3</td>
<td>0.9999</td>
</tr>
<tr>
<td>Ctrl3</td>
<td>5.4</td>
<td>5.57</td>
<td>4.1</td>
<td>0.9989</td>
</tr>
<tr>
<td>Ctrl4</td>
<td>20.0</td>
<td>8.88</td>
<td>3.8</td>
<td>0.9910</td>
</tr>
<tr>
<td>Ctrl5</td>
<td>20.0</td>
<td>5.97</td>
<td>2.6</td>
<td>0.9832</td>
</tr>
<tr>
<td>Ctrl6</td>
<td>16.0</td>
<td>7.55</td>
<td>3.7</td>
<td>0.9999</td>
</tr>
<tr>
<td>Ctrl7</td>
<td>16.2</td>
<td>7.97</td>
<td>3.8</td>
<td>0.9989</td>
</tr>
<tr>
<td>Ctrl8</td>
<td>20.0</td>
<td>6.72</td>
<td>2.9</td>
<td>0.9970</td>
</tr>
<tr>
<td>Ctrl9</td>
<td>20.0</td>
<td>4.41</td>
<td>1.9</td>
<td>0.9869</td>
</tr>
<tr>
<td>Patient 2</td>
<td>18.3</td>
<td>4.50</td>
<td>2.0</td>
<td>0.9982</td>
</tr>
<tr>
<td>Patient 1a</td>
<td>3.2</td>
<td>0.51</td>
<td>0.4</td>
<td>0.9989</td>
</tr>
<tr>
<td>Patient 1b</td>
<td>4.2</td>
<td>0.67</td>
<td>0.5</td>
<td>0.9981</td>
</tr>
<tr>
<td>Ctrl + sertraline</td>
<td>20.0</td>
<td>0.95</td>
<td>0.4</td>
<td>0.9995</td>
</tr>
</tbody>
</table>

|                          |                 |                                   |                    |                                               |
| Statistical analysis Vmax and V at mean Km concentration |                 |                                   |                    |                                               |
| Control mean           | 15.14           | 6.37                              | 3.24               |                                               |
| Control Standard Deviation | 1.42            | 0.67                              |                    |                                               |
| Control mean -1SD      | 4.95            | 2.57                              |                    |                                               |
| Control mean -2SD      | 3.52            | 1.90                              |                    |                                               |

Table 14. SERT Km and Vmax were calculated for all samples using non-linear regression methods.
3.4 Serotonin transporter deficiency

3.4.1 Introduction to SERT sequencing

Serotonin transporter deficiency was demonstrated by low serotonin uptake into platelets in patients. This can be one of the causes of both central (CSF) serotonin metabolite and peripheral serotonin (platelet serotonin) deficiency in the patients, and would be suggestive of a primary serotonin transporter deficiency in this family. Although the SERT dysfunction was not demonstrated with strong evidence in the proband’s sister, meaning that there might be multiple causes for the low serotonin levels, we have proceeded with the serotonin transporter gene sequencing. The \textit{SLC6A4} gene is on chromosome 17: 41,649 bp, has 630 amino acids, and 14 exons.

3.4.2 Methods

Genomic DNA was isolated from blood with DNA Extraction Kit (Qiagen) and all exons and adjacent regions of the \textit{SLC6A4} gene were amplified by standard PCR with Taq DNA Polymerase and dNTP mix from Invitrogen. Oligonucleotide primer pairs flanking all 14 exons were designed (exons 1, 2 and 14 divided in 2 parts were used) All primers described in this section and sections 3.6.2 and 3.7.1 were synthesized in the NAPS Unit, a core facility at Michael Smith Laboratories at UBC, and they are detailed in (Table 15 and Table 16).
Table 15. SERT forward primers
Universal primer is highlighted. “a” and “b” indicates that the exon was divided into two parts for sequencing.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>5′- TGTAAAACGACGGCCAGTATGACACCTCCTCGGCTCTCC -3′</td>
</tr>
<tr>
<td>1b</td>
<td>5′- TGTAAAACGACGGCCAGTTGGGTGGGATTTCCTCTGTGGA -3′</td>
</tr>
<tr>
<td>2a</td>
<td>5′- TGTAAAACGACGGCCAGTGACATAATCTGTCTTCTCCTGCA -3′</td>
</tr>
<tr>
<td>2b</td>
<td>5′- TGTAAAACGACGGCCAGTGCCAGACATTGCCCAGGTCCA -3′</td>
</tr>
<tr>
<td>3</td>
<td>5′- TGTAAAACGACGGCCAGTGACATAATCTGTCTTCTCCTCAA -3′</td>
</tr>
<tr>
<td>4</td>
<td>5′- TGTAAAACGACGGCCAGTGCCAGACATTGCCCAGGTCCA -3′</td>
</tr>
<tr>
<td>5</td>
<td>5′- TGTAAAACGACGGCCAGTGACATAATCTGTCTTCTCCTCAA -3′</td>
</tr>
<tr>
<td>6</td>
<td>5′- TGTAAAACGACGGCCAGTGCCAGACATTGCCCAGGTCCA -3′</td>
</tr>
<tr>
<td>7</td>
<td>5′- TGTAAAACGACGGCCAGTGACATAATCTGTCTTCTCCTCAA -3′</td>
</tr>
<tr>
<td>8</td>
<td>5′- TGTAAAACGACGGCCAGTGCCAGACATTGCCCAGGTCCA -3′</td>
</tr>
<tr>
<td>9</td>
<td>5′- TGTAAAACGACGGCCAGTGACATAATCTGTCTTCTCCTCAA -3′</td>
</tr>
<tr>
<td>10</td>
<td>5′- TGTAAAACGACGGCCAGTGCCAGACATTGCCCAGGTCCA -3′</td>
</tr>
<tr>
<td>11</td>
<td>5′- TGTAAAACGACGGCCAGTGACATAATCTGTCTTCTCCTCAA -3′</td>
</tr>
<tr>
<td>12</td>
<td>5′- TGTAAAACGACGGCCAGTGCCAGACATTGCCCAGGTCCA -3′</td>
</tr>
<tr>
<td>13</td>
<td>5′- TGTAAAACGACGGCCAGTGACATAATCTGTCTTCTGAGTTG -3′</td>
</tr>
<tr>
<td>14a</td>
<td>5′- TGTAAAACGACGGCCAGTGCCAGACATTGCCCAGGTCCA -3′</td>
</tr>
<tr>
<td>14b</td>
<td>5′- TGTAAAACGACGGCCAGTGCCAGACATTGCCCAGGTCCA -3′</td>
</tr>
</tbody>
</table>

Table 16. SERT reverse primers
Universal primer is highlighted. “a” and “b” indicates that the exon was divided into two parts for sequencing.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>1b</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>2a</td>
<td>5′- GTGAAAGACGGAAGGCAAGAAAGAGCTACGGCTAGGTGGA -3′</td>
</tr>
<tr>
<td>2b</td>
<td>5′- GTGAAAGACGGAAGGCAAGAAAGAGCTACGGCTAGGTGGA -3′</td>
</tr>
<tr>
<td>3</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTTCA -3′</td>
</tr>
<tr>
<td>4</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>5</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>6</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>7</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>8</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>9</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>10</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>11</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>12</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>13</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>14a</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
<tr>
<td>14b</td>
<td>5′- CAGGAAACAGCATTGACACCGGCAACACCTGCTCTTCTT -3′</td>
</tr>
</tbody>
</table>

Promoter region was also sequenced with the following oligonucleotide primer pairs (universal primer highlighted) (Table 17 and Table 18).
Table 17. Forward SERT promoter primers

<table>
<thead>
<tr>
<th></th>
<th>5’-</th>
<th>3’-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TGTAAAACGACGGGTGCAGCTTAAAGGCGGCCGAATACGGGTAGGGTGCAAGGAAGG-3’</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>TGTAAAACGACGGGTGCAGCTTAAAGGCGGCCGAATACGGGTAGGGTGCAAGGAAGG-3’</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>TGTAAAACGACGGGTGCAGCTTAAAGGCGGCCGAATACGGGTAGGGTGCAAGGAAGG-3’</td>
<td></td>
</tr>
</tbody>
</table>

Table 18. Reverse SERT promoter primers

<table>
<thead>
<tr>
<th></th>
<th>5’-</th>
<th>3’-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAGGAAACAGCTATGACCCCGACATCTGCGGAGGGTTA</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CAGGAAACAGCTATGACCCCGACATCTGCGGAGGGTTA</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CAGGAAACAGCTATGACCCCGACATCTGCGGAGGGTTA</td>
<td></td>
</tr>
</tbody>
</table>

Amplification was carried out using an initial denaturation of 94°C for 2 minutes, 60°C, 62°C, 65°C or 68°C for 90 minutes, 72°C for 2 minutes, 29 cycles from step 1, and a final elongation of 72°C for 8 minutes. All PCR products were purified using QIAquick PCR Purification Kit Protocol with microcentrifuge (QIAGEN) and visualized by running 5 µL of product on 6% acrylamide gels. Purified products were sent directly to the DNA Sequencing Core Facility at the Center for Molecular Medicine and Therapeutics (Vancouver). The SERT promoter region was sequenced with Long PCR method using Expand Long Template PCR System Kit (from Roche - for long >12 kB amplification). Amplification was carried out using an initial denaturation of 94°C for 2 minutes, 90°C for 30 seconds, 60°C, or 65°C for 1 minute, 68°C for 15 seconds, repeat cycles 9 times from step 2, then again 94°C for 30 seconds, 600 or 650 for 1 minute, then 68°C for 5 minutes + 20 seconds/cycle, repeat 19 times to step 5, cycles from step 1, and a final elongation of 68°C for 20 minutes. DNA purification from the 1% acrylic gel was done with QIAquick Gel Extraction Kit Protocol (QIAGEN).

3.4.3 Results

Sequencing result was negative for mutations in the SLC6A4 gene. The two affected sisters were homozygous for the 12VNTR allele in intron 2. The promoter region sequencing did reveal that both siblings carry the LL (homozygous) allele (16 repeats, long allele of 5HTTLPR). Both these variants are associated with higher expression of the gene. These studies were done in relation with the psychiatric literature, showing a positive correlation between the short allele (S) and anxiety related traits. In our patient’s transcriptome analysis we have found no difference between the expression of the three samples, but we have not
genotyped the unaffected sister. The mother and affected brother also did not have their SERT gene sequenced.

3.5 Vesicular monoamine transporter deficiency

Vesicular monoamine transporter deficiency that can cause both serotonin and dopamine deficiency, has been recently described with a completely different clinical phenotype, and this gene was not sequenced.

3.6 Serotonin developmental pathway. SPT5

The next logical step building the hypothesis in finding the molecular etiology for the systemic serotonin deficiency was to look in the serotonin developmental pathway. SPT5 and FEV were the two genes in the pathway described in Figure 8 that are not yet associated with human disease, although FEV has been proposed to be candidate gene for infants with sudden unexpected death. These two genes have been sequenced.

3.6.1 Introduction

Considering that it can affect transcription of multiple genes, and its deficiency could potentially also cause systemic serotonin deficiency, SPT5 was proposed as a candidate gene, and was sequenced. (chromosome 19, 3710 bp, 1087 amino acids, 30 exons).

3.6.2 Methods

The 30 exons were captured by Long PCR (Roche) with previously described method, flanking 7 larger sized sequences. Primers were synthesized as described in section 3.4.2. Details on forward and reverse primers with universal primer highlighted are in Table 19 and Table 20.

<table>
<thead>
<tr>
<th></th>
<th>Forward Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’- TGTAAAACGACGCGCCAGTGGGAGATGGCAGTGCTGTCG-3’</td>
</tr>
<tr>
<td>2</td>
<td>5’- TGTAAAACGACGCGCCAGTTTCCCAGAGACAGCCAGCTCCC-3’</td>
</tr>
<tr>
<td>3</td>
<td>5’- TGTAAAACGACGCGCCAGTGGACAGACAAGCTAGGCGTGGA-3’</td>
</tr>
<tr>
<td>4</td>
<td>5’- TGTAAAACGACGCGCCAGTGGCTGCCAAGGGTGAGTGTGTC-3’</td>
</tr>
<tr>
<td>5</td>
<td>5’- TGTAAAACGACGCGCCAGTGCTCTAGCTGGCCCTGACATGCACCACA-3’</td>
</tr>
<tr>
<td>6</td>
<td>5’- TGTAAAACGACGCGCCAGTTGTACCATGACATGACATGCACCACA-3’</td>
</tr>
<tr>
<td>7</td>
<td>5’- TGTAAAACGACGCGCCAGTCCACCCTGACATGACATGCACCACA-3’</td>
</tr>
</tbody>
</table>

Table 19. SPT5 forward primers
3.6.3 Results

SPT5 gene sequencing results revealed only common variants from the SNP database with allele frequency greater than 1%.

3.7 Serotonin developmental pathway. FEV

3.7.1 Methods

The three exons of the FEV (PetI) gene (chromosome 2, 1879 pb, 238 amino acids) were also captured by Long PCR (Roche) and subjected to sequencing as before, using the primers synthesized as described in section 3.4.2, and are shown in Table 21 and Table 22.

<table>
<thead>
<tr>
<th></th>
<th>5’-CAGGAAACAGCTATGACCCCCGCCACATCTGCGGAGGGTTA-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5’-CAGGAAACAGCTATGACCCCCGCCACATCTGCGGAGGGTTA-3’</td>
</tr>
<tr>
<td>3</td>
<td>5’-CAGGAAACAGCTATGACCCCCGCCACATCTGCGGAGGGTTA-3’</td>
</tr>
<tr>
<td>4</td>
<td>5’-CAGGAAACAGCTATGACCCCCGCCACATCTGCGGAGGGTTA-3’</td>
</tr>
<tr>
<td>5</td>
<td>5’-CAGGAAACAGCTATGACCCCCGCCACATCTGCGGAGGGTTA-3’</td>
</tr>
<tr>
<td>6</td>
<td>5’-CAGGAAACAGCTATGACCCCCGCCACATCTGCGGAGGGTTA-3’</td>
</tr>
<tr>
<td>7</td>
<td>5’-CAGGAAACAGCTATGACCCCCGCCACATCTGCGGAGGGTTA-3’</td>
</tr>
</tbody>
</table>

Table 20. SPT5 Reverse primers

<table>
<thead>
<tr>
<th></th>
<th>5’-AAGATGCAGATAACGCAGCCTGGA-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5’-TCCTGACTTGGGCTCTATGG-3’</td>
</tr>
<tr>
<td>3</td>
<td>5’-AACTAGAACCCAGGCAAACTGGCT-3’</td>
</tr>
</tbody>
</table>

Table 21. FEV forward primers

<table>
<thead>
<tr>
<th></th>
<th>5’-CATTACAATCGGCCCTCCCATGCAA-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5’-CTTGGCCTGCAACTCTTTTC-3’</td>
</tr>
<tr>
<td>3</td>
<td>5’-CGTCCCCATCGCATAAAAGTCTCCA-3’</td>
</tr>
</tbody>
</table>

Table 22. FEV reverse primers

3.7.2 Results

Sequencing of the FEV gene showed no variants.
3.8 Cytoskeleton

3.8.1 Introduction SERT protein quantification

Serotonin transporter function was low in patients but there were no pathogenic mutations found with the gene sequencing. To further investigate the cause of the low function of the serotonin transporter, we performed protein quantification with western blot (WB).

3.8.2 Methods

Patient and control platelet samples were pelleted, washed and protein content was measured with the Lowry method. The running gels were made in the lab: 8% polyacrylamide lower gel, and 4% upper gel. Western blots using the serotonin transporter antibody ST (H-115) sc-13997, (Rabbit polyclonal IgG Santa-Cruz) were done with the wet tank transfer technique using an ice-cooling unit, and running the transfer for 1 hour at 100 V constant. Labeling was done with HRP-colour developing substrate; reading with chemiluminescence. Serial dilutions of the primary antibody (between 1:2000 – 1:500) have been tried with unsatisfying results, showing faint bands, or additional nonspecific bands. A new antibody from Abnova has been also tried (Mouse polyclonal antibody, H00006532-A01), with very similar results. Although the bands were faint there was no difference noted between patient and control samples. Cultured fibroblasts were pelleted, sonicated and protein content measured with the same Lowry method. Western blots were run using both antibodies against SLC6A4 but no bands were obtained on the membranes.

As these standard Western blot methods with commercial antibodies gave unsatisfactory results, collaboration was sought out with one of the internationally renowned laboratories working on serotonin transporter research, the Blakely Lab at Vanderbilt University, Nashville, USA. Platelet rich plasma (PRP) was sent to the Blakely Lab from Vancouver from 7 controls and the two affected sisters. Their lab used their own serotonin transporter antibody that they developed in rabbits. Platelets were pelleted from PRP and solubilized in PBS with 1% Triton-X. The supernatant fraction was designated “Triton-soluble (TS)”. The pellet was solubilized in RIPA buffer, resulting in the extract called “Cytoskeleton (CS)” fraction. SERT is a membrane skeleton associated protein when activated, and upon inactivation internalizes. SERT function is dependent on its subcellular localization.
3.8.3 Results

The results showed more abundant SERT in patient samples than in controls (Figure 22). This could potentially be explained with the LL variant in the promoter, which has been associated with higher expression of the protein.

**Figure 22.** SERT WB in proband, proband’s sister and two controls (protein size markers Kb are shown on the left)
TS – Triton-soluble platelet fraction, CS – cytoskeleton fraction

Control WB was run with actin, to assess sample integrity. The patient samples had lower quantity of actin than controls (Figure 23).
Western Blot – β-Actin

Figure 23. WB of actin in proband, proband’s sister and two controls. (protein size markers Kb are shown on the left)

3.9 Proteomic analysis

3.9.1 Introduction

To further investigate the cause of the low expression of actin in the patient samples, proteomic analysis was performed.

3.9.2 Methods

The cytoskeleton (CS) and Triton-soluble (TS) fractions were resolved by denaturing polyacrylamide gel electrophoresis and visualized with Coomassie Blue staining, for in gel protein detection (Figure 24).
Figure 24. Coomassie stained gel of CS and TS fractions in 2 patients and 2 controls. The arrows to bands 1, 2, 3, 5, 6 and 7 are pointing to the control samples because those were diminished, and were in insufficient quantity for mass analysis in the patient’s samples.

Bands 1 through 6 were cut out and analyzed by Mass Spectrometry (MS) for protein identification. Using 2D gel protein electrophoresis with an antiphospho-serine, -threonine antibody would have given more information on protein phosphorylation.

3.9.3 Results

The results of the proteins identified in the protein gel are shown in Table 23. Proteins that had a different band on the patient’s lanes were mostly proteins associated with cytoskeleton.

<table>
<thead>
<tr>
<th>Band</th>
<th>Protein identified with MS</th>
<th>Protein identified with MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Myosin</td>
<td>intracellular soluble protein</td>
</tr>
<tr>
<td>2</td>
<td>Filamin-A or Talin-1</td>
<td>cytoskeletal protein</td>
</tr>
<tr>
<td>3</td>
<td>Talin-1</td>
<td>cytoskeletal protein</td>
</tr>
<tr>
<td>4</td>
<td>Serum albumin</td>
<td>intracellular soluble protein</td>
</tr>
<tr>
<td>5</td>
<td>Cofilin-1</td>
<td>cytoskeletal protein</td>
</tr>
<tr>
<td>6</td>
<td>Myosin-9</td>
<td>intracellular soluble protein</td>
</tr>
<tr>
<td>7</td>
<td>Complement component-3</td>
<td>intracellular soluble protein</td>
</tr>
</tbody>
</table>

Table 23. Proteins identification with Mass Spectometry.
3.10 Cytoskeleton component analysis

3.10.1 Introduction

To clarify the question of why do patient samples have more SERT and less actin, and why are cytoskeleton proteins different, I went to the Blakely Lab to perform further experiments, and spent 12 weeks there (over the period of 2 years).

The previous WB’s for SERT were repeated, and Western Blots for cytoskeleton proteins were run.

3.10.2 Methods

Same materials and methods of platelet fractionation were used. PRP was thawed and kept on ice thereafter. First spin was at 5000 g x 3-4 minutes. Pellet was washed with 1xPBS 10 ml. The spin was repeated at same rpm x 2min, and washed with 1xPBS again twice. The pellet was lysed then with 1% TritonX (overnight on shaker in the cold room). Lysate was centrifuged at max speed (16,000 g) at 4\(^0\) x10 min. The pellet was dissolved in RIPA buffer for obtaining the cytoskeleton (CS) fraction. The supernatant was taken off and subjected to ultracentrifugation (100,000 g) to obtain the supernatant Triton soluble (TS) fraction, and the pellet, the membrane skeleton (MS) fraction. In the CS fraction interactions between cytoskeletal proteins provide resistance against disruption by nonionic detergents, such as Triton X-100. The MS fraction contains the transmembrane protein assembly (receptors, ion channels, membrane associated proteins). The TS fraction contains the majority of intracellular proteins.

Protein content was measured using the PIERCE protein concentration bicinchoninic acid (BCA) spectrophotometric assay.

Another sample was run simultaneously called “Total platelet sample” which was obtained from platelet pellet by solubilizing it in 1% SDS. Primary antibody was Monoclonal antibody SERT “51” (51mAb) – mouse (Blakely Lab) (1:1000).

Western blotting was done with the wet tank transfer technique, with the “sandwich” transfer for 18 hours at 20 V constant overnight in the cold room. The running gels were premade commercial gels: Nu-Page 4-12% Bis-Tris Gel (1.0 mm x 10 well). Labeling was done using primary antibodies for actin, vinculin, talin, and cofilin from Sigma-Aldrich (monoclonal anti β-actin (1:50,000), vinculin, talin and cofilin, (1:2000 each) product #F3165, V9264,
Secondary antibody was used from Jackson ImmunoResearch Laboratories (Goat antimouse GAM 50% calf serum; 1:10,000). Western Lightning ECL mix was used for developing on X-ray films.

3.10.3 Results

SERT protein was expressed in patient platelet TS and total platelet samples in increased quantities (Figure 25).

Two control samples from individuals who took 5-hydroxytryptophan supplements for two months prior to blood collection were later added, and those results looked similar to the other control samples (Figure 26).

All WB experiments were repeated and the results were reproducible.

Actin, vinculin, talin and coflin were decreased in patient’s platelet Triton-soluble fraction (Figure 27). These cytoskeleton proteins were selected because they were diminished in the previous proteomic analysis. The mild decrease in the total platelet actin in one experiment could be secondary to the fact that due to aggregations, the platelet pellet was not dissolved sufficiently. The actin level was identical with controls in the proband’s fibroblasts, and it was used as control for the antibody and WB method.

Figure 25. SERT WB in proband and proband’s sister and two control platelet samples (two bands in platelet samples may indicate non-specific crossreactivity with the antibody).

Figure 26. SERT WB in proband and proband’s sister and two controls on treatment with 5HTP.
3.10.4 Conclusion

The platelet fractionation studies showing increased SERT expression in the Triton-soluble fraction, which is the intracellular pool, suggests that there is reduced functional membrane SERT, meaning that cytoskeleton aggregates trap the transporter protein inside the cell. The increased expression of SERT in total platelets could be partly explained by the SERT gene promoter LL genotype. In addition there was a reduction of actin and other cytoskeletal proteins in the Triton-soluble fraction, but unchanged in total expression. This suggests also presence of cytoskeleton aggregates inside the cells, in the cytoskeleton fraction.
3.11 Proteomics GeLC-MS

3.11.1 Methods

Total platelet samples were run on SDS-PAGE gel and stained with Coomassie-blue and silver, to assess for the different protein content. MS analysis was performed of select individual gel bands with semi quantitative GeLC-MS proteomic analysis.

3.11.2 Results

Coomassie and silver stained gels showed difference in protein amount between patients’ and controls’ total platelet samples (Figure 28 and Figure 29).

Proteomic analysis of 4 columns (Ctr1, 5HT1, Pt1, Pt2) showed proteins that were different in patient samples. Protein bands were cut out from gel, were identified by mass, and are shown in Table 24. Proteins identified with MS to be diminished in quantity in patient samples were mainly cytoskeleton-associated proteins. This analysis was done on all proteins in a section of the column, and the previous proteomic analysis was done on individual selected bands, that were noted diminished in patient samples.

Coomassie stain total platelets

![Coomassie stain total platelets](image)

Figure 28. Coomassie stain of total platelets in patients and controls. Ctr1, Ctr2 – regular controls; 5HTP1, 5HTP2 – controls taking 5HTP for 2 months prior to sampling; pt1, pt2 – proband and proband’s sisters’ samples (protein size given in kb along the left margin). Bands labeled with arrow and questionmark are the ones that were different in patients.
Silver stain total platelets

Figure 29. Silver stained gel of total platelets in patients and controls. The upper part of the gel labeled between the arrows was cut and sent for proteomic analysis. Individual bands of proteins identified are not shown. Ctr1, Ctr2 – regular controls; 5HTP1, 5HTP2 – controls taking 5HTP for 2 months prior to sampling; pt1, pt2 – proband and proband’s sisters’ samples

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓</td>
<td>Filamin 1</td>
</tr>
<tr>
<td>↓</td>
<td>Filamin B</td>
</tr>
<tr>
<td>↓</td>
<td>Actin</td>
</tr>
<tr>
<td>↓</td>
<td>Talin 1</td>
</tr>
<tr>
<td>↓</td>
<td>α-actinin 1</td>
</tr>
<tr>
<td>↓</td>
<td>α-actinin 4</td>
</tr>
<tr>
<td>↓</td>
<td>Vinculin</td>
</tr>
</tbody>
</table>

Table 24. Proteins identified with MS to be diminished in quantity in patient samples (only the cytoskeleton proteins that were low are shown in the table)

3.12 Platelet imaging

3.12.1 Introduction

To look for cytoskeleton aggregation, gold-labeling of actin in patient and control platelet samples was performed.
3.12.2 Methods

Platelet samples from deceased proband and a control were fixed in 4% formaldehyde solutions and kept frozen at -70°C. Before analysis, platelet samples were thawed, pelleted and mixed with warm 5% low melting agarose/PBS solution. The solidified agarose gel with imbedded platelets then was taken to the UBC Bioimaging Facility for actin labeling and imaging.

3.12.3 Results

Gold labeled actin was aggregated in small clumps in patient platelet samples compared with control platelets (Figure 30 and Figure 31).

Figure 30. Control platelet gold labeled actin. Arrow indicates gold labeling of actin.
The platelet studies have shown a difference in subcellular localization of the SERT and some selected cytoskeleton proteins, suggesting that the cytoskeleton is aggregated, trapping some membrane associated proteins, such as SERT internalized. This has been also shown in the electronmicroscopy imaging with gold labeled actin being aggregated in the patients platelets. This could explain in part the biochemical phenotype with the low serotonin uptake in the proband and affected sister, although there were some differences in the SERT uptake between the sisters. We could hypothesize that the transporter is not associated with the membrane, so it is not functional. Through similarity we could also hypothesize that some ion channels and
ATPases, such as the voltage gated calcium channel and Na/K ATPase could be also trapped inside the cell, although no experiments were done to demonstrate this. These membrane associated proteins have a role in the pathology of hemiplegic migraines. However no genetic cause was postulated on why would there be cytoskeleton changes and differences in compartmentalization of certain proteins in platelets, which lead the research to its next phase of generating a hypothesis with Whole Exome Sequencing.
Chapter 4: Hypothesis generating research

In order to generate a hypothesis regarding the disorder in this family we performed Whole Exome Sequencing (WES). It covers all exons of the nuclear DNA and some mitochondrial DNA.

4.1 Whole exome sequencing

4.1.1 Introduction

To generate a hypothesis in this family we sent WES on the affected proband, the unaffected sister and their mother. The other two affected siblings and the father were added later.

4.1.2 Methods

The analysis was done in the Molecular Genetics Lab at the Vanderbilt University in Nashville, with HiSeq 2000 Sequencing System by Illumina. Capture was done with Agilent SureSelect 38M.

4.1.3 Results

There was good coverage with total reads between 300M and 450M per exome (>95% mapped). Snp analysis was done with Exome Variant Server and we filtered out all variants with >1% frequency. Based on the hypothesis driven from the four-generation pedigree suggestive of an autosomal dominant (AD) inheritance mode with variable expressivity and gender variation, we have chosen to look for AD inherited candidate genes. For the autosomal dominant transmission mode there were 111 non-synonymous variants found in 92 genes, with 7 novel (missense) and 3 nonsense variants. (Table 25).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Variation</th>
<th>Functions, disease relevance</th>
<th>dbSNP</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTTNBP2NL</td>
<td>I273T</td>
<td>Serine/threonine phosphatase, linked to cerebral cavernous malformations</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>DPP10</td>
<td>T456M</td>
<td>Serine protease, linked to asthma and inflammatory disease</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>KLHL21</td>
<td>D322Y</td>
<td>Uncharacterized, part of Kelch-like protein family, associated with cancer, familial hyperkalemic hypertension, AD retinitis pigmentosa</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>SRRM2</td>
<td>P630Q</td>
<td>Pre-mRNA splicing</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>PLIN3</td>
<td>K421E</td>
<td>Interacts with mannose-6-phosphate receptors associated with lypodystrophy, sebaceous adenoma</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>EPCAM</td>
<td>R173H</td>
<td>Epithelial cellular adhesion molecule, associated with high risk of cancer</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>CXorf66</td>
<td>F213L</td>
<td>Secretory glycoprotein, associated with different cancers</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>DNAH3</td>
<td>W3024X</td>
<td>Dynein heavy chain 3, axonemal, present in cilia (sperm, lungs)</td>
<td>rs148298506</td>
<td>0.001/1</td>
</tr>
<tr>
<td>STAP2</td>
<td>Y169X</td>
<td>Src-type non-receptor tyrosine kinase, associated with cancer</td>
<td>rs79657645</td>
<td>0.005/11</td>
</tr>
<tr>
<td>LDLR</td>
<td>C27X</td>
<td>Low density lipoprotein receptor, associated with familial hypercholesterolemia</td>
<td>rs2228671</td>
<td>0.058/126</td>
</tr>
</tbody>
</table>

Table 25. Candidate genes from AD model

In the autosomal recessive transmission mode there were 203 variants found in 147 genes, with 6 candidate genes remaining after filtering for predicted pathogenicity and frequency. These were in the following genes: GSTM3 (glutathione S-transferase mu, associated with susceptibility to adverse reactions to carcinogens and toxins), KRTAP13-2 (high sulfur keratin associated protein essential for the formation of a rigid and resistant hair shaft), HLA-DRB1 (HLA class II beta chain paralog, with role in the immune system), VPS13B (vacuolar protein sorting 13 homolog B, causing Cohen syndrome), and UBE2NL (ubiquitin conjugating enzyme E2N-like, this truncating mutation was found with high frequency in cohort of patients studied for X-linked mental retardation and also in their controls). These genes were not considered consistent with the clinical presentation of the patients in this study.

Looking at the function of the genes, known genetic disorders, and the frequency of the variants, we filtered out genes until only two candidate genes in the AD transmission mode remained. These were both novel variants, chr16:20,976,134C>T (axonemal dynein DNAH3 W3024X; a nonsense mutation that changes the amino acid Tryptophan to stop codon).
variant was later found in control population in the 1000G project, and later was disregarded from our study. The other variant was the chr16:2,812,418C>A (SR protein SRRM2 P630Q; a missense mutation that changes the amino acid Proline into Glutamine).

Sanger sequencing of DNAH3 to confirm the findings was performed in the Nashville Vanderbilt University Molecular Lab (Figure 32).

![DNAH3 Sanger sequencing](image)

**Figure 32.** DNAH3 Sanger sequencing
A Proband, B Proband’s sister, C Mother, D Unaffected sister, E Brother, F Father. The mutated nucleotide is marked with an arrow.

Cytoplasmic dynein is the most important molecular motor transporting a wide range of cargoes. It has been shown to have role in axonal retrograde transport and studies revealed evidence of association with pathology in neurodegenerative diseases. Because axonemal dynein is only expressed in flagella and cilia, and in the brain perhaps only expressed in the chorionic plexus, and because there was a SNP report a few months after our WES results, which reported the W3024* variant in a healthy control, we have eliminated this gene from the candidate gene list. It was reported as rs148298506 by 1000Genome in North American population with allele frequency 0.001.

SRRM2 Sanger sequencing was also done in the Nashville Vanderbilt University Molecular Lab (Figure 33).
SRRM2 gene (OMIM#606032) with Genomic coordinates: 16p.13.3; chr16: 2802329-2821410 in GRCh37 (Genome Reference Consortium), contains 20,210 bases and 15 exons, RefSeq accession: NM_016333, RNA accession: NM_016333.3, RNA size: 9379, protein Q9UQ35 (UniProt), protein molecular mass 300kDa. It is a 2,296 amino acid protein rich in serine (S), arginine (R), and proline (P), containing numerous SR peptides and 2 long polyserine domains. Its subcellular location is in the nucleus speckle.

The chr 16:2,812,418C>A variant is a novel variant, not found in any of the SNP databases. Polyphen predicted the P630Q variant as a possibly damaging variant with a score of 0.952, and SIFT predicted it 100% damaging. For the P630Q variant, introducing a glutamine instead of the proline the PepTool program predicts introduction of α-helices into the protein, causing 3D structural changes to it. The proline at the 630 position is a proline dependent kinase phosphorylation site. It is an area rich in SR/RS dipeptide repeats. Using the SMART domain database (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de/) the amino acids between position 563 and 682 are in the CTD (carboxy terminal domain), homologous to the C-terminal domain of the transcription elongation factor protein Spt5. This
domain in Spt5 is necessary for binding to Spt4, to form the functional complex that regulates early transcription elongation by polymerase II. The complex may be involved in pre-mRNA processing through its association with mRNA-capping enzymes.

At chr16:2812939 there was another variant p.P804T, rs2240140, with an allele frequency in the general population of 0.423/923, present in the two affected sisters, and absent in the brother and mother and unaffected sister. It resides in a significant repetitive sequence [(SX)SSPE(PK) consensus sequence occurring ten or more times in the ORF-Open Reading Frame]. It is predicted to be a benign polymorphism by Polyphen tool. Both variants are in the 355 amino acid-length Arginine-rich region that comprises the amino acids between positions 462-816. Both variants reside in the longest exon of the gene.

### 4.1.4 p.P630Q variant prediction

The Department of Computational Medicine and Bioinformatics at the University of Michigan offers a free service of predicting models for proteins, called I-TASSER (Iterative Threading ASSEMBly Refinement). The I-TASSER server has been developed to generate automated full-length 3D protein structural predictions. The server is free to download for academic purposes and gives several predicted models, not only one, for each protein that has no crystal structure (http://zhanglab.ccmb.med.umich.edu/I-TASSER/download/). Two different predicted models of the wildtype SRRM2 protein and two for the P630Q variant are shown in Figure 34. for comparison. Although the exact site of the altered amino acid cannot be readily indicated in this figure, the overall change to the structure relative to the two possible predicted normal structures is obvious. Proline is a unique amino acid with the side chain connected to the protein backbone twice, forming a five-membered ring. It is found in very tight turns in protein structures and it is unable to occupy other main-chain conformations like the other amino acids. It can also function to introduce kinks in α-helices, since it is unable to adopt a normal helical conformation. Proline does not substitute well with other amino acids.
Figure 34. I-TASSER program predictions for SRRM2 protein structure.  
(a) and (c) Two different predicted structures for wild-type protein (630P), (b) and (d) Two different predicted structures for protein with 630Q variant.
The proline is conserved at the position 630 in different human SRRM2 protein isoforms and multiple species (Figure 35 and Figure 36).

**Figure 35.** SRRM2 protein alignment for position p630
Proline is conserved

**Figure 36.** SRRM2 nucleotide alignment block 2812413-2812444 (32 bp)
The nucleotide that is part of the triplet forming Proline (the highlighted C), is conserved at position 2812418
4.1.5 SRRM2 protein quantification

Quantification of the SRRM2 protein was done in fibroblasts with WB, which showed no difference between patient and control. The antibody used was SRm300 (C-9) sc-390315, Mouse monoclonal antibody Santa-Cruz (Figure 37).

![Western blot of SRRM2 protein in a control and proband’s affected sister. WB fibroblasts SRRM2 (300 kDa) present in both control and patient. Lane 1 - control sample Lane 2 – proband’s sister (protein size in Kb given along left margin)](image)

**Figure 37.** Western blot of SRRM2 protein in a control and proband’s affected sister. WB fibroblasts SRRM2 (300 kDa) present in both control and patient. Lane 1 - control sample Lane 2 – proband’s sister (protein size in Kb given along left margin)

4.2 SRRM2 and SRRM1 protein interaction

4.2.1 Introduction

To look at the protein-protein interaction between SRRM1 and SRRM2 (Figure 10 where SRm300 is SRRM2 and SRm160 is SRRM1), Co-immunoprecipitation studies were performed.

4.2.2 Method

Co-IP SRRM2/SRRM1 was performed with the DMP (Dimethyl pimelimidate) crosslinking protocol, using dynabeads (Life Technologies Dynabeads Protein A). Nuclear extraction from fibroblasts was done with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology).
4.2.3 Results

Immunoprecipitation reaction using SRRM1 as the primary target and SRRM2 as secondary target (interacting protein) showed that SRRM1 interacts with SRRM2 in patient fibroblast samples. There was a presence of a band in the negative control (IgG) indicating that the Co-IP technique needs to be refined, and for this reason figure of the results is not shown.

4.2.4 Discussion

This result is not unexpected, because the P630Q variant is found in a region of the protein that is not present in the yeast homolog, Cwc21, being a shorter protein, and this is the original organism where the interaction of SRRM1 and SRRM2 were described by Blencowe. The full list of the proteins that interact with SRRM2 is not known, but it is predicted to be many (Figure 11), and without knowing the full nature of these proteins, no further Co-IP experiments were conducted.

4.3 Transcriptome analysis

4.3.1 Introduction

To further characterize the effect of the SRRM2 variant, and its effect on splicing or transcription, RNA sequencing was performed on the proband’s affected sister, brother and unaffected sister.

4.3.2 Methods

RNA sequencing on Illumina platform was done by the Perkin Elmer Biotechnology Company. Data analysis was done with GeneSifter (Analysis Edition). RNA was extracted from blood. Considering the alterations in platelet cytoskeleton, it was considered to be an appropriate source for analysis. The unaffected sister was used as control for the two affected siblings. No other controls were used in this experiment.

4.3.3 Results

There were no changes noted in splicing but a significant number of genes were considered to be potentially involved by being up- or downregulated.
Significant changes in expression were noted in genes belonging to ontologies of cytoskeleton organization, synaptic transmission, biogenic amine transport and transport, amongst others. Results comparing the three samples are summarized in the Heat-Map analysis below. It shows the differential gene expression by displaying values in a coloured format. Each coloured cell in the heat map represents the gene expression value for a probe in a sample. The largest gene expression values (upregulated genes) are displayed in red (hot), the smallest values (downregulated genes) in green (cool).

Pathway analysis, or analysis of sets of genes differentially expressed in distinct phenotypes, was done for actin cytoskeleton organization, amine biogenesis, amine transport, synaptic transmission, serotonin receptor signaling, and G-protein-coupled receptor expression (Figure 38, Figure 39 and Figure 40).

Figure 38. Heat-map analysis of actin cytoskeleton organization (column on left) and amine transport (column on right) pathway genes. Column 1 - unaffected sister (used as control); Column 2 – proband’s affected sister; Column 3 - affected brother. (red upregulated, green downregulated genes)
Figure 39. Heat-map analysis of synaptic transmission pathway (column on left) and G-protein-coupled receptor (column on right) genes
Column 1 - unaffected sister (used as control); Column 2 – proband’s affected sister; Column 3 - affected brother (red up-, green down-regulated genes)

Figure 40. Heat-map analysis of serotonin receptor signaling pathway genes
Column 1 - unaffected sister (used as control); Column 2 – proband’s affected sister; Column 3 - affected brother (red up-, green down-regulated genes)
4.3.4 Individual gene expression

Individual gene comparisons showed significantly overexpressed \textit{ATP1A2}, Filamin 1, \textit{MAOB}, and \textit{NOTCH3} genes amongst many others, and significant downregulation of \textit{CACNA1A}, 5HT$_{2A, 3B}$, and $\alpha_2$-actinin genes amongst others. A sample of the figure is illustrated through the \textit{ATP1A2} and \textit{CACNA1A} genes (Figure 41).

![Figure 41](image.png)

\textbf{Figure 41.} Comparison in gene expressions between unaffected control and affected siblings. (a) Na+/K+ ATPase (upregulated); (b) P/Q type voltage gated Ca++ channel (downregulated)
1 – unaffected sister, 2 – proband’s affected sister, 3 – proband’s brother

A list of selected genes considered being important for the clinical phenotype and pathogenesis is summarized in Table 26, Table 27, Table 28, Table 29 and Table 30.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold increase</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulin $\beta_2$</td>
<td>2.5-6.0</td>
<td></td>
</tr>
<tr>
<td>Tubulin $\beta_2A$</td>
<td>2-6</td>
<td></td>
</tr>
<tr>
<td>Talin 2</td>
<td>2-3</td>
<td></td>
</tr>
<tr>
<td>Actin 1$\alpha$</td>
<td>4-6</td>
<td></td>
</tr>
<tr>
<td>Profilin 1,2,3,4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Spectrin 2</td>
<td>4-8</td>
<td></td>
</tr>
<tr>
<td>Kinesin 4A</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Filamin A</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Actin related protein (Arp)</td>
<td>1.5-2.5</td>
<td></td>
</tr>
<tr>
<td>ARPM11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>FILIP1</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>KIF1A</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Destin</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Titin</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

\textbf{Table 26.} Overexpressed cytoskeleton genes.
Summary of selected underexpressed cytoskeleton genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold increase</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinin α2, 3</td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>Agrin</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Gelsolin</td>
<td></td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table 27. Underexpressed cytoskeleton genes.

Selected serotonin related genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold increase</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERT</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5HT&lt;sub&gt;2A&lt;/sub&gt; receptor</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>5HT&lt;sub&gt;3B&lt;/sub&gt; receptor</td>
<td></td>
<td>5-10</td>
</tr>
<tr>
<td>TPH2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TPH1</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>MAOA</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MAOB</td>
<td>4-9</td>
<td></td>
</tr>
</tbody>
</table>

Table 28. Serotonin related genes.

Familial hemiplegic migraine related genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold increase</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACNA1A</td>
<td></td>
<td>3-5</td>
</tr>
<tr>
<td>ATP1A2</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Table 29. FHM related genes.

Other selected genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold increase</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN2B</td>
<td></td>
<td>2-10</td>
</tr>
<tr>
<td>GTPCH1</td>
<td></td>
<td>1.5-5</td>
</tr>
<tr>
<td>NOTCH3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>NOS2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Rho GTPase activating protein</td>
<td>3-10</td>
<td></td>
</tr>
<tr>
<td>DNAH3</td>
<td></td>
<td>3-6</td>
</tr>
</tbody>
</table>

Table 30. Other selected genes.

4.3.5 Discussion and summary

Finding the different expression levels of certain genes in well-known neurodegenerative pathways and ontologies is the most significant finding of this research project. Although there were no significant differences in splicing events, this does not rule out that the variant in our candidate gene is responsible for the variation in gene expression, as it was similar to the C. elegans, where complete knock-out of the gene caused larval arrest, but transcriptome analysis
in RNA interference (RNAi) animals showed a global decrease in transcript levels. The multiple roles of arginine-serine rich splicing factors in RNA processing was demonstrated by Sanford et al. \(^{141}\), who showed that a subset of SR proteins shuttle continuously between nucleus and cytoplasm. Shuttling SR proteins may have additional roles in mRNA transport, localization, stability and regulation of translation. An unexpected function of SR proteins was their role in nonsense-mediated decay. Shuttling proteins also regulate the translation of specific mRNA targets. Elucidation of these targets would help understand how SR proteins influence gene expression.

The P630Q variant in \(SRRM2\) did not change the protein amount or mRNA expression, but could have altered protein function, such as interaction with other SR proteins. There may have been also be mis-splicing that generates unstable mRNAs (eg premature stops that may be subject to nonsense-mediated mRNA decay as explained above).

Finding previously differences in the cytoskeleton organization in platelets lead to the specific selection of pathways and ontologies, taking back the research aspect to hypothesis driven. In the cytoskeleton organization pathway there were a large number of genes significantly up- and down-regulated. It has already been demonstrated that there is cytoskeleton aggregation in platelets that traps the proteins, which have to be expressed on the membrane in order to be functional, such as SERT, the Na\(^+/K^+\) ATPase, the ion channels and various receptors. This has been also suggested by the histochemistry findings on the post-mortem brain autopsy in the deceased proband, with accumulation of synaptophysin that can be suggestive of axonal transport block, due most likely to neurofilament aggregation. The cytoskeleton aggregation could be the cause of the systemic serotonin deficiency, if SERT is trapped inside the cells.

Other important ontology pathways are the synaptic transmission and monoamine metabolism pathways. Finding a significantly upregulated \(MAOB\) gene might give further explication for the low serotonin metabolite levels, as it could be that serotonin is broken down in excess in neurons. The downregulated serotonin receptors could also give insight to the response to treatment. This could be considered important if looking for a group of other ND disorders that could be responsive to serotonin therapy.

From the FHM genes the \(ATP1A2\) gene was significantly up-regulated and the \(CACNA1A\) gene was downregulated several folds. Both of these findings could be at the basis of the hemiplegic migraines and the associated coma and cerebellar atrophy. Interestingly in 94
**elegans** the function of the Ca$^{2+}$ channel ortholog, UNC-2 is required for bilateral expression of TPH1, suggesting some further explanation for the serotonin deficiency, by diminished synthesis.

The *NOTCH3* upregulation was quite significant at 6-fold, along with other neurodevelopmental genes such as *Hox3* suggesting a developmental small spinal cord rather than atrophy.

Interestingly there were oncogenes in the pathways that were differentially regulated, but there was a difference between the two affected siblings. We had no transcriptome analysis on the proband who had the tumours to be able to compare.

The cause for neurodegeneration could be the sum of the effects of all the up- and down-regulated genes. We have looked at the genes that are known to cause single gene ND disorders, and found a few genes from most of the ND disease groups. Some were up-regulated, others were down-regulated. There is still much to learn about the molecular pathomechanism of neurodegeneration, but future research might shed light on certain genes of “key importance” that are at the core of these degenerative processes. This could be further assessed by testing the enrichment of pathways for all differently expressed genes by using bioinformatics tools, but this requires a larger number of samples to be able to run for example in the Vanderbilt tool [http://bioinfo.vanderbilt.edu/webgestalt/](http://bioinfo.vanderbilt.edu/webgestalt/). Such an approach is a future goal if more families with similar clinical and biochemical presentation can be identified.
Chapter 5: Conclusions, perspectives, future directions

5.1 Summary of research

Our quest for solving the etiology of this family’s neurodegenerative disease started with hypothesis driven research, built on a careful clinical, radiological, and biochemical differential diagnosis. It has lead to the bench, looking for further biochemical evidence and individual candidate gene sequencing. As we have ruled out many hypotheses along the way and whole exome sequencing (WES) technology became more readily available, we continued with a hypothesis generating research, finding a candidate gene through WES. To validate the candidate gene variant we performed whole RNA sequencing, and again used hypothesis driven approach when selecting the ontology pathways to be analyzed. By treating the patients throughout the research with 5-hydroxytryptophan we have closed the loop of translational research taking it back to the bedside (Figure 42).

Figure 42. Translational model of current research
5.2 Weighing the evidence of SRRM2 mutation being causative

The P630Q variant is in the arginine-serine repetitive sequence of the SRRM2 gene, does not affect protein expression, and does not affect protein concentration. The only plausible hypothesis is that it affects its post-translational modification, hence protein function. The most important function of the SR proteins is protein-protein interaction in the spliceosome formation. The complete list of proteins interacting with SRRM2 is not known yet, and finding these interactors should be the first future project. Perturbation of these protein-protein interactions could lead to dysfunction in mRNA transport, localization, stability, regulation of translation, nonsense-mediated decay, and translation of specific mRNA targets.

This family had members in three generations affected, but to a different degree, supporting the autosomal dominant inheritance pattern with variable expressivity. The two most severely affected sisters carry another rare missense variant (P804T) in the SRRM2 gene. The P630Q novel variant could be necessary but not sufficient for the severe clinical phenotype, and the P804T variant could be a modifier. Considering the two male family members not having any problems with weakness, one could hypothesize a gender difference also. Cooper at al. wrote an excellent overview on genotype/phenotype correlation, bringing up multiple examples of Mendelian disorders where genotype alone is not predictive of phenotype. We have done a microarray for detection of copy number variants and found no abnormalities in the proband, but other intragenic or other gene-gene interactions cannot be ruled out, one can only speculate on their role.

The difference in gene expression pattern supports the hypothesis that the SRRM2 protein, which is at the core of the spliceosome and participates in pre-mRNA splicing, mRNA transport and regulation of translation stands at the basis of his family’s neurodegeneration. This is also supported by the fact that no splicing event dysregulation was found in the C.elegans RNAi model, only differences in gene expression pattern (Table 31).
Table 31. Pros and cons of SRRM2 being causative

<table>
<thead>
<tr>
<th></th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene level</strong></td>
<td>P630Q predicted to be damaging (SIFT, PolyPhen)</td>
<td>Missense variant</td>
</tr>
<tr>
<td></td>
<td>Variant is novel</td>
<td></td>
</tr>
<tr>
<td><strong>Protein level</strong></td>
<td>Proline highly conserved, and difficult to substitute</td>
<td>No difference in protein level on WB</td>
</tr>
<tr>
<td></td>
<td>Predicted 3D structure changes configuration with the introduction</td>
<td>No crystal structure</td>
</tr>
<tr>
<td></td>
<td>of the variant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SR protein-protein interaction not characterized</td>
<td></td>
</tr>
<tr>
<td><strong>RNA level</strong></td>
<td>Gene transcription variation comparable to C. elegans knock-down</td>
<td>No abnormal splicing events</td>
</tr>
<tr>
<td></td>
<td>model</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypothesis driven pathway analysis suggestive of biochemical and</td>
<td>Pathways for all differently expressed genes not available until</td>
</tr>
<tr>
<td></td>
<td>molecular biology findings</td>
<td>further samples and cases identified</td>
</tr>
</tbody>
</table>

The cytoskeleton aggregation could explain some aspects of the phenotype, through the hypothesis of trapping membrane proteins inside the cell, away from their functional cellular domain. Such proteins would be SERT, the P/Q voltage gated calcium channel and Na/K ATPase, the first explaining the low systemic serotonin, the latter two being affected in patients with familial hemiplegic migraines. Low serotonin could be also the consequence of the excessive metabolism by an upregulated MAOB enzyme. Actin and actin related proteins form the cellular cytoskeleton and its dynamic properties are provided by phosphorylation and dephosphorylation of certain signaling cascade molecules, which could be up- or downregulated as a consequence of transcriptional irregularities caused by a dysfunctional SRRM2 protein. Cytoskeleton aggregation could also explain the axonal transport deficiency seen on the proband’s postmortem brain histopathology, with the synaptophysin accumulation, ultimately explaining the “dying back” distal neuropathy and degeneration phenomenon.

There could be a specific affinity or predilection for the transcriptional differences in some gene ontology clusters, and this needs to be subject for future research.

The overall conclusion of neurodegeneration can be hypothesized with the diagram in Figure 43, considering that in the present case we have found evidence of cytoskeleton aggregates, axonal transport defect and difference in gene expression pattern, all of these being described to cause neurodegeneration.
5.3 Proposed molecular model for explanation of neurodegeneration

Many neurodegenerative disorders (ND) share common pathologic mechanisms and overall have been found ultimately to have changes in different gene expressions. How these differences in gene expression occur is not yet understood, but these single gene disorders can be great models for complex neurodegenerative pathophysiology. Researchers are searching for all those common genes that are mostly affected in a number of different ND disorders, trying to look for answers on how these changes in expression levels occur and what, or if, there is a common answer, or common denominator in the molecular pathogenesis. Some ND diseases have been considered to have no single, but “complex” genetic etiology, based on the fact that genes considered risk factors are numerous and have complex patterns of interactions. This hypothesis can be challenged with the present family, revealing a previously described variance in certain gene expression but having a single hypothetical molecular basis at the centre of the RNA spliceosome. The transcriptome analysis did not demonstrate significant changes in accuracy of splicing events, but demonstrated significant changes in transcription levels of genes in well recognized ND disease pathways (i.e. cytoskeleton, synaptic transmission) and at individual gene levels, as discussed in the previous chapter. This has also been observed in the knock-down *C. elegans* model organism.

The changes in the transcription levels are not associated with sequence changes in the genes themselves, as the results of WES analysis would have shown this, and therefore looking
at a common factor later in the process of production of viable mRNA such as the SRRM2 protein, at the core of the spliceosome complex, is a reasonable hypothesis. Our patients had several clinical features mimicking well-known ND disorders, and have been found to have up- and down-regulated genes that can cause these individual disorders (Figure 44). This could be interpreted as simplistic and most likely the explanation is much more complex. We suggest that the differentially expressed genes are at the basis of causing the neurodegeneration.

**Figure 44.** Differentially expressed neurodegenerative genes
Up- and down-regulated genes in our patients that have been involved to cause known ND disorders. Genes in green are down-regulated >2.0 times, genes in red are up-regulated >2.0 times.

### 5.4 Treatment model

One of the major aspects in this family’s unique neurodegenerative course was the significant improvement in lower limb strength with serotonin replacement therapy with 5HTP/carbidopa. This could be explained with several aspects of serotonin’s role in the body as
described before, through modulation of synaptic transmission and as an intracellular signaling molecule. The large number of serotonin receptors in the central nervous system and in the spinal cord, are good evidence of the important role this neurotransmitter has in the body. Modulation of $K^+$ and voltage-gated $Ca^{2+}$ channels will change glutamate and GABA mediated neurotransmission, but will also act directly on some of the ion channels that are directly symptom causing in the patients in this family. (i.e.$Ca^{2+}$ and $Na^+$ channels encoded by $CANCA1A$, $SCN1A$).

Hyposerotonininemia has been linked to several neurodegenerative disorders, such as PD, ALS and other motor neuron diseases. The intracellular signaling properties of serotonin are carried out through the G-proteins that are coupled to 5HT receptors and act on protein kinase C or A (PKC, PKA) amongst other signaling molecules, changing ultimately the nuclear gene transcription, but acting also directly on the status of the cytoskeleton (Figure 45).

Figure 45. Serotonin's effect on synaptic modulation and intracellular signaling
The successful treatment of the weakness in this family should not only encourage clinical trials of N=1 as proposed, but perhaps could include other selected ND patient groups that mainly affect lower limb weakness.

5.5 Limitations and benefits of the study

Single case studies always have limitations, but having multiple affected members of the same family can add value to genetic research. Although the exact mechanism of how the variants in the SRRM2 protein affect the function of the protein and ultimately the spliceosomal integrity and transcription, remain undetermined, this family case study has taught me how to build a research plan and a hypothesis starting from the bedside, leading to translational research, and then coming back in full circle to the bedside with a treatment plan. Furthermore it has proven that the hypothesis of differential transcription of “key” genetic/ontological pathways stays at the basics of neurodegeneration. Perhaps studying further the clusters of genes up- and down-regulated in this case will lead to better understanding of the pathophysiology of neurodegeneration overall. It is possible that the spliceosome and various transcription factors have a central role in this process. Understanding the pathophysiology will ultimately lead to development of better treatment strategies. This study also highlights the importance and usefulness of current, new methods of genetic analysis and bioinformatics tools, such as WES and transcriptome analysis. The latter will be most likely used more frequently in the future in genetic research.

5.6 Future directions

Finding the SRRM2 associated proteins with further Co-Ip studies could be the first step in future research. Expanding the list of knock-out SRRM2 gene model organisms beyond the yeast and C. elegans, towards more complex animal models, such as the zebrafish or mice, would most likely shed light to a very intricate genetic mechanism involving the spliceosome, pre-mRNA splicing and transcription. The SRRM2 gene is much larger in these organisms than the yeast and C. elegans ortholog and the part that is added to the human protein is the one that has importance in binding to other spliceosomal component proteins. Having a viable higher organism knock-out model would allow not only further genetic research into the
pathophysiology of neurodegeneration, but most importantly would give us more answers to the mechanism of action of treatment with serotonin.

Finding other families with the same molecular defect would be another strategy to expand and further characterize the clinical, biochemical and molecular phenotype and response to treatment. This is already being done with publications and networking with national and international colleagues, through presentations at meetings or through web-based networking.

Serotonin has been always considered a naturopathic supplement, and has never been manufactured in pure pharmaceutical powder form. Current research focuses on new drug development acting on individual types of serotonin receptors, and not on serotonin itself.

I believe that developing a drug similar to Sinemet, which is a combination of L-dopa/carbidopa, but with 5-hydroxytryptophan in association with carbidopa, the latter to block the peripheral AADC enzyme, should be one of the first steps in future therapeutic research.
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Appendix A

Protocol Safety and Efficacy Assessment for carbidopa
Clinical N=1 Trial Application
Principal Applicants:

Gabriella Horvath, MD (Div. Biochemical Diseases, Department of Pediatrics)
Bruce Carlton, PhD (Department of Pharmacology)

Title of proposal:

N=1 clinical trial for efficacy and safety of carbidopa treatment in two siblings with systemic serotonin deficiency

Condition:

Systemic serotonin deficiency in siblings with hemiplegic migraines, spinal cord atrophy, myoclonic seizures, progressive spastic paraparesis, and mood disorder, with a spliceosome gene (SRRM2) mutation causing modifications in multiple gene expressions.

Case presentations

We follow two siblings affected with hemiplegic migraines, myoclonic seizures and spinal cord atrophy. The sister is 25 years old and the brother is 21 years old. They had a sister, who passed away at the age of 22 years who was affected with the same condition, but a much more severe form. The sister is more affected then her brother, she has progressive spastic paraparesis and severe lower limb weakness. They both have mood disorder, the sister has depression and the brother anxiety disorder. Their seizures are controlled with valproic acid. The sister is on supplementation with 5-hydroxytryptophan and carbidopa (to block the peripheral AADC activity) and her strength improved.
dramatically. The brother is on no medication other than the valproic acid. His strength is still good.

Diagnosis and Background

The underlying genetic diagnosis that has been recently characterized, is a mutation in the *SRRM2* gene, encoding a small protein in the spliceosome machinery, affecting pre-mRNA splicing. RNA splicing is a highly conserved process in eukaryocytes that transforms primary transcripts, or pre-mRNAs through the removal of intronic sequences. This process is accomplished by the spliceosome, which is a large dynamic RNA-protein complex. SR proteins are an evolutionarily conserved family characterized by an RNA recognition motif and a region rich in arginine and serine dipeptides (RS domain), which are important for protein-protein interactions. It has been shown that the *C. elegans* ortholog of human SRRM2 has a role in transcription. RNA transcriptome analysis in the siblings revealed many differently expressed genes compared to control. Some of the underexpressed genes explain the low systemic serotonin levels.

Serotonin (5-HT) is a monoamine neurotransmitter synthesized in neurons of midbrain raphe nuclei in CNS and in enterochromaffin cells (ECC) in the gut. The rate limiting step in serotonin synthesis is catalyzed by two genetically distinct isoforms of tryptophan hydroxylase. *TPH2* is preferentially expressed in the brain, while *TPH1* is expressed peripherally and in the pineal gland. Serotonin is stored intracellularly in vesicles and granules. Re-uptake of neuronal serotonin across presynaptic plasma membrane is mediated by the serotonin transporter SERT, a member of the SLC6 sodium dependent solute transporter family (*SLC6A4*). SERT is highly expressed in platelets that do not synthesize serotonin, but store serotonin produced by the ECC. Serotonin (5HT) plays diverse roles in the regulation of cardiovascular function, breathing, circadian rhythm, sleep, appetite, mood and learning. It influences a wide range of psychiatric disorders and it is not surprising that 5HT has been targeted for many pharmacologic approaches to managing depression and anxiety. Further, 5HT and its numerous receptor subtypes are involved with behavioral and neuroendocrinological processes that provide life-preserving responses to stress. Serotonin plays an important role in vascular resistance and blood
pressure control, and a functional serotonin transporter polymorphism has been associated with migraine \cite{74,165}. In addition, prior to assuming its role as a neurotransmitter, 5HT acts as a trophic signal in the developing brain by directing neural ontogeny of the 5HT and other neurochemical systems. As a developmental signal, 5HT regulates cell migration, axonal outgrowth and synaptogenesis, and as a coordinating mediator of intercellular communication. Therefore it is conceivable that changing levels of central 5HT during prenatal development may alter subsequent structure and function of the serotonergic system \cite{166}. Serotonin is present in the anterior horn motor neurons and in the lateral part of the substantia gelatinosa, where it is associated with terminals of descending fibers, modulating segmental reflexes and nociception \cite{78,167}.

Actual problem

The sister has progressive lower limb weakness, which has been responsive to 5-hydroxytryptophan and carbidopa administration. This could be explained by restoration of serotonin inhibition of glycinergic inhibitory neurons in the spinal cord, in the pontine reticular, and respiratory muscles \cite{85}.

Objective

Utilizing Serotonin as glycinergic inhibitor and hence activator of motor neurons in spinal cord, we will demonstrate improvement of strength in C.H. We will add carbidopa to prevent 5HTP decarboxylation in the periphery and to enhance transport into the brain \cite{89,168}.

Pharmacokinetics of 5-hydroxytryptophan (5HTP) and carbidopa has been studied in the past.

5HTP alone can have serious, disturbing GI side effects: nausea, vomiting, diminished appetite. Blocking the peripheral AADC enzyme will block peripheral decarboxylation and most of the drug will be transported in the brain. Carbidopa alone or in combination has not been shown to cause serious side effects.
Side effects

Serious side Effects of 5HTP: allergic reaction: hives; difficulty breathing; swelling of your face, lips, tongue, or throat.
Less serious side effects may include: stomach pain, nausea, vomiting, gas, diarrhea, loss of appetite.

Supply

5-hydroxytryptophan is often sold as an herbal supplement. There are no regulated manufacturing standards in place for many herbal compounds and some marketed supplements have been found to be contaminated with toxic metals or other drugs. The supplement will be purchased from a reliable source (BC Children’s Hospital Outpatient Pharmacy) to minimize the risk of contamination.

Anticipated side effects of 5HTP: above mentioned GI side-effects. These side effects should subside with the addition of carbidopa. Carbidopa alone has not been associated with any side-effects (only in combination with levodopa).

Protocol of trial

<table>
<thead>
<tr>
<th></th>
<th>Assessment</th>
<th>Assessment</th>
<th>Assessment</th>
<th>Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sister</td>
<td>Baseline (on 5HTP+carbidopa treatment)</td>
<td>1 month off treatment</td>
<td>2 weeks on 5HTP alone</td>
<td>1 month on both 5HTP +carbidopa</td>
</tr>
<tr>
<td>Brother</td>
<td>-</td>
<td>Baseline</td>
<td>2 weeks of 5HTP alone</td>
<td>1 month on both 5HTP +carbidopa</td>
</tr>
</tbody>
</table>

Tools for assessment:

a. Performance based tests to assess physical function and lower body strength (validated tests)\(^{169}\)
i. 30 second chair test
ii. stair climb test
iii. 40 m fast paced walk test
iv. timed up and go test
v. 6 min walk test

b. Manual muscle testing (performed by Physiotherapist)\textsuperscript{170}
c. DASS (Depression Anxiety and Stress Scale)\textsuperscript{171}
d. GI dysfunction:
   i. Nausea
   ii. Vomiting
   iii. Cramping, gas, diarrhea
   iv. Constipation

e. Frequency, duration and severity of hemiplegic migraine episodes

Adverse events

In case of adverse events, these will be reported to Health Canada, by the attending physicians.

Written consent from patient’s family will be obtained (Appendix B).
Appendix B

Consent forms

DEPARTMENT OF PEDIATRICS
2D19 – 4480 OAK STREET, VANCOUVER, BC  V6H 3V4
TEL: 604.875.3177  FAX: 604.875.2890

PARTICIPANT INFORMATION AND CONSENT FORM (ADULT)

Open label N=1 clinical trial for assessing effectiveness and safety of 5HTP+carbidopa in a patient with systemic serotonin deficiency

Principal Investigator: [Redacted]

Co-Investigator: [Redacted]
When we say “you” in this consent form, we mean you; “we” means the doctors and other staff.

WHY YOU ARE BEING INVITED TO TAKE PART IN THIS STUDY

Doctors and scientists at the University of British Columbia are investigating the treatment of systemic serotonin deficiency with 5-hydroxytryptophan (5HTP)+carbidopa. This study is a single case study.

You are being invited to take part in this research because you have systemic serotonin deficiency.

YOUR PARTICIPATION IS VOLUNTARY

Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in this study. Before you decide, it is important for you to understand what the research involves. This consent form will tell you about the study, why the research is being done and what will happen during the study. The possible benefits, risks and discomforts are described.

If you wish to participate, you will be asked to sign this form. If you do decide to take part in this study, you are still free to withdraw at any later time and without giving any reasons for your decision.

If you do not wish to participate, you do not have to provide any reasons for your decision not to participate nor will you lose the benefit of any medical care to which you are entitled or are presently receiving.

Please take time to read the following information carefully and to discuss it with your family, friends and doctor before you decide.

WHO IS CONDUCTING THE STUDY?

This study is being conducted by the Principal Investigator, Dr. Gabriella Horvath and Co-Investigator, Dr. Bruce Carleton, in the Division of Biochemical Diseases and Department of...
BACKGROUND

You have been found to have low levels of platelet and spinal fluid serotonin. Serotonin is a chemical that has important role in the brain but also in the rest of your body. It has been related to mood problems, migraines, normal gut function, and many of the autonomic functions your body does without your will, such as sweating, and temperature regulation. Your serotonin transporter that takes up serotonin from plasma into platelets to store it was also found to be non-functional. Extensive genetic research has identified the underlying genetic problem to be in a gene that encodes a protein that regulates how well is information encoded in genes transformed into protein. There seems to be a problem in translating this information, which ultimately affects many different proteins in your body, including how your body produces serotonin, and transports it in and out of the cell. Because your serotonin transporter does not work properly we cannot increase the serotonin levels with our standard medication, which is a serotonin re-uptake inhibitor (SSRI), and we will give you 5-hydroxytryptophan (serotonin precursor that your body can transform into serotonin). 5HTP alone gets broken down in the gut by an enzyme, and in order to get as much serotonin precursor to your brain as possible, we will add carbidopa to it, which blocks this enzyme. These two medications have no interaction with the valproic acid that you take for your seizures.

WHAT IS THE PURPOSE OF THE STUDY?

The main purpose of this study is to see if addition of 5HTP+carbidopa has a beneficial effect on your leg strength, mood problem, and your overall wellbeing.

WHO CAN PARTICIPATE IN THE STUDY?

Currently only you fit the criteria for participation.

WHO SHOULD NOT PARTICIPATE IN THIS STUDY?

No one else is eligible for this study.
WHAT DOES THIS STUDY INVOLVE?
If you decide to participate in this study the following will happen:

- The principal investigator or the other study investigators will explain the study to you, including the risks of taking 5HTP+carbidopa, answer your questions and ask you to sign the consent form.
- Your participation will consist of four outpatient physiotherapy assessments, for a total of about 5 hours. These will be done at baseline, before the beginning of treatment, 2 weeks after you take 5HTP alone, 1 month after you’ve been taking both 5HTP+carbidopa and at the end of the trial at 3 months on both 5HTP+carbidopa. The assessment will consist of standardized manual strength and performance assessments, and you will be also asked to fill out a short standardized depression/anxiety questionnaire at home at your leisure.
- We will ask you to fill out a short clinical symptom record after each period, and also record any potential side effects that you experience.

Data and results will be collected identified only by a study number and will be destroyed after 25 years. Only the study investigators and their students and staff will have access to the data.

WHAT ARE THE BENEFITS AND RISKS OF PARTICIPATING IN THIS STUDY?
Participation in this study has the potential benefit of improvement in your strength, mood problems, migraines, sleep and energy problems. We also hope that the information learned from this study can be used for future benefit of others with serotonin deficiency.

Carbidopa is an investigational drug, approved by FDA in the US, but obtained only with permission from Health Canada. Carbidopa alone has not been associated with any adverse effects (only in combination with levodopa, when the adverse effects are those of levodopa)

5HTP is a non-pharmaceutical naturopathic drug that is available at Health Food stores. Our pharmacy at BC Children’s Hospital is purchasing it from a well known and trusted source, and the 5HTP is produced by a company named Medisca. We use it routinely in patients who have biopterin synthesis defect, a cofactor that is required in the brain to produce
neurotransmitters, such as serotonin. Known adverse effects include allergic reactions: hives; difficulty breathing; swelling of your face, lips, tongue, or throat. This event would be treated with standard allergy medications.

Less serious side effects may include: stomach pain, nausea, vomiting, gas, diarrhea or loss of appetite. These side effects will be treated with antinausea medications, such as Gravol or Ondansetron, adding an antacid, and making sure 5HTP is taken together with meals.

Results will be shared with Health Canada, with de-identification, only with a study number.

WHAT ARE THE ALTERNATIVES TO PARTICIPATING IN THIS STUDY?

The only alternative to participating in this study is non-participation. Your non-participation will in no way affect your health care.

WHAT HAPPENS IF I DECIDE TO WITHDRAW MY CONSENT TO PARTICIPATE?

Your participation in this research is entirely voluntary. You may withdraw from this study at any time. You are not required to provide any reason for your decision. If you decide to enter the study and to withdraw at any time in the future, there will be no penalty or loss of benefits to which you are otherwise entitled, and your future medical care will not be affected. However all data collected and already analyzed during your enrollment in the study will be retained for analysis.

If you decide to withdraw, please contact [Contact Information].

The study doctor(s)/investigators may decide to discontinue the study at any time, or withdraw you from the study at any time, if they feel that it is in your best interests.

CAN I BE ASKED TO LEAVE THE STUDY?

The investigators may decide to stop you from taking part in this study at any time. You could be removed from the study for reasons related only to you (for example, if you moved and were no longer contactable).
AFTER THE STUDY IS FINISHED

The study investigators will explain the results to you. You will have the option of whether or not you agree to let us disclose this information to your family physician. These options will be discussed with you when results are communicated.

WHAT WILL THE STUDY COST ME?

Research participants should not incur any personal expenses as part of this study. There is no payment for participation.

Signing this consent form in no way limits your legal rights against the investigators, or anyone else, and you do not release the study doctors or participating institutions from their legal and professional responsibilities.

COMMERCIALIZATION

It is possible that commercialized products may eventually derive from this research. However, you will not receive any financial benefits or intellectual property rights from your participation.

CONFIDENTIALITY OF RECORDS AND SAMPLES

Your confidentiality will be respected. However, research records and medical records identifying you may be inspected in the presence of the Investigator or his designate by the representatives of Health Canada and the Children’s and Women’s Health Centre Clinical Research Ethics Board and for the purpose of monitoring the research. No information or records that disclose your identity will be published without your consent, nor will any information or records that disclose your identity be removed or released without your consent unless required by law.

You will be assigned a unique study number as a subject in this study. Only this number will be used on any research-related information collected about you during the course of this study, so that your identity [i.e. your name or any other information that could identify you] as a subject in this study will be kept confidential. Information that contains your identity will
remain only with the Principal Investigator and/or designate. The list that matches your name to the unique identifier that is used on your research-related information will not be removed or released without your consent unless required by law.

Your rights to privacy are legally protected by federal and provincial laws that require safeguards to insure that your privacy is respected and also give you the right of access to the information about you that has been provided to the sponsor and, if need be, an opportunity to correct any errors in this information. Further details about these laws are available on request to the Principal Investigator.

Final results of this study will be published in scientific journals or presentations. All efforts will be taken so you cannot be identified.

OBTAINING ADDITIONAL INFORMATION

The study is under the direction of Dr. Gabriella Horvath. You are encouraged to ask questions at any time during the study. In the event that you have questions about the study please call (604) 875-2880. If you have any concerns or complaints about your rights as a research subject and/or your experiences while participating in this study, contact the Research Subject Information Line in the University of British Columbia Office of Research Services by e-mail at RSIL@ors.ubc.ca or by phone at 604 822 8598 (Toll Free:1-877-822-8598)
Participant Consent

- I am satisfied that the information contained in this consent form was explained to me to the extent that I am able to understand it.
- I hereby give consent to participate in this study.
- I acknowledge having received a signed and dated copy of this consent form.
- I have read and understood the participant information and consent form.
- I have had sufficient time to consider the information provided and to ask for advice if necessary.
- I have had the opportunity to ask questions and have had satisfactory responses to my questions.
- I understand that all information collected will be kept confidential.
- I understand that my participation in this study is entirely voluntary and that I am completely free to refuse to participate or to withdraw from this study at any time without changing in any way the quality of care that I am receiving.
- I understand that I am not waiving any of my legal rights as a result of signing this consent form.
- I authorize access to my medical record as described in this consent form.
- I understand that there is no guarantee that this study will provide any direct or immediate benefits to me.
- I authorize access to my health records and samples as described in this consent form.

The participant and the investigator are satisfied that the information contained in this consent form was explained to the participant to the extent that he/she is able to understand it, that all questions have been answered, and that the subject consents to participating in the research.

________________________________________
Participant's Name (please print)

________________________________________
Participant’s Signature      ______________________
Date