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**Optical imaging and automated behavior as tools for the
diagnostic monitoring of brain function in
neurodevelopmental disorders**

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Abstract

Neurodevelopmental disorders (NDDs) are a class of variegated conditions affecting 1-2% of the general population, characterized by cognitive impairment, behavioral abnormalities, sensory and motor changes, and speech and language deficits resulting as developmental disorders of the brain and nervous system.

The pathological mechanism is not fully understood and may be related to hereditary or environmental factors. NDDs occurs during infancy, and in most cases, has a chronic progression over a lifetime. NDDs encompasses a heterogeneous group of disorders including, but not limited to, CDKL5 deficiency disorders, creatine transporter deficiency syndrome, Krabbe disease, and Angelman syndrome. These conditions have a robust, known genetic origin and a devastating impact on patients and their families. Recently, it has been indicated that brain energetics might concur to the pathophysiology of these diseases as a common mechanism.

Unfortunately, there is no cure for these disorders; however, various mutant mice are now available, resulting in good models for evaluating the pathophysiology of the disease. Moreover, some therapies are beginning to be tested at the preclinical level and the evaluation of the efficacy of these experimental

therapies needs a robust, translational, unbiased, and quantitative biomarker to non-invasively and quantitatively monitor brain function. To respond to this demand, I used physiological techniques, such as intrinsic optical signal (IOS) imaging and VEP, to investigate the cortical functions of these disorders. Monitoring visual function proved to be a precise tool, highlighting alterations in the intrinsic spectral properties of oxygenated and deoxygenated hemoglobin in the primary visual cortex in each of the strains investigated. Moreover, employing this biomarker to monitor brain function during treatment reveals a solid ability to accurately detect modifications of visual responses; overall, these findings identify novel, translational and non-invasive biomarkers for the analysis of brain function in NDDs.

As mentioned above, NDDs have a wide range of cognitive and behavioral manifestations and their assessment represents a primary point for physicians, psychologists and researchers. Mouse models recapitulate these behavioral and cognitive alterations; however, classical paradigms used in neuroscience to evaluate these aspects are subjected to different biases. Here, I implemented, validated, and applied in C57BL/6J and CDKL5 mice a novel automated and 3D printed tool, based on appetitive conditioning protocols that perform operant conditioning on freely moving mice. Thanks to the complete automaticity of the test and the ability to accurately detect parameters, its results as a robust behavioral tool, applicable to monitor longitudinally the effectiveness of treatments and washout effects in preclinical, and possibly clinical, studies in NDDs.

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Chapter 1

Introduction

1.1 Neurodevelopmental disorders

In the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), the term "neurodevelopmental disorders (NDDs)" refers to: "a group of conditions with onset in the developmental period. The disorders typically manifest early in development, often before the child enters grade school, and are characterized by developmental deficits that produce impairments of personal, social, academic, or occupational functioning. The range of developmental deficits varies from very specific limitations of learning or control of executive functions to global impairments of social skills or intelligence. The neurodevelopmental disorders frequently co-occur; for example, individuals with autism spectrum disorder often have intellectual disability (intellectual developmental disorder), and many children with attention-deficit/hyperactivity disorder (ADHD) also have a specific learning disorder. For some disorders, the clinical presentation

includes symptoms of excess as well as deficits and delays in achieving expected milestones. For example, autism spectrum disorder is diagnosed only when the characteristic deficits of social communication are accompanied by excessively repetitive behaviors, restricted interests, and insistence on sameness” (APA American Psychiatric Association 2013).

The definition proposed by DSM-5 includes a plethora of heterogeneous conditions that are distinctly classified to identify groups of patients requiring distinct services and are not meant to reflect biologically distinct entities. The diagnostic criteria for NDDs are based on a constellation of behaviors/symptoms that are specified in DSM and are also coded in the WHO international classification of disease (ICD) (Ismail and Shapiro 2019).

If on the one hand, it is necessary to establish criteria that would allow an adequate classification of the disorders, on the other hand, the extreme variability and overlapping of conditions that may co-occur, makes the diagnosis extremely complicated. For these reasons new constructs to implement the diagnosis were proposed. Some authors suggested that NDDs should be framed as a spectrum with varying severity of impairment in a continuum from minimal brain/cerebral dysfunction to most severe forms of impairment, where all domains of development can be affected with varying degrees of involvement (Capute and Palmer 1980). Christopher Gillberg proposed the Early Symptomatic Syndromes Eliciting Neurodevelopmental Clinical Examinations (ESSENCE) in order to describe the multiple areas of dysfunction especially in preschoolers (Christopher Gillberg 2010) and deficits in attention, motor control, and perception (DAMP), which encompasses ADHD and developmental coordination disorder (C. Gillberg 2003). Informed by new genetic discoveries of shared copy number variants and single-gene mutations, other authors proposed that the term ‘developmental

brain dysfunction' denoting that NDDs disorders should be considered as a group when evaluated under the genetics lens (Moreno-De-Luca et al. 2013). A further factor contributing to the difficulty in diagnosis is the heterogeneous etiology of NDDs, including genetic as well as nongenetic factors. Epidemiologic studies have attempted to establish the association of various environmental factors with NDDs, such as maternal health conditions, perinatal infection, malnutrition, trauma, and many others (Chaste and Leboyer 2012). The genetic contribution to NDDs is variable, but reports of increased rates of concordance for NDDs among twins and family members support a high degree of heritability (Gentner and Leppert 2019). There is substantial genetic heterogeneity, as there are an enormous number of individually rare genetic conditions that present with an NDDs as a prominent feature. These include chromosome copy number variants (CNVs) and single-gene disorders. Moreover, non-genetic factors may modify the manifestation of pathological features, thus resulting in a multifactorial condition. Other mechanisms include epigenetic alterations or a polygenic contribution with multiple common variants or polymorphisms collectively resulting in an NDDs (Siu and Weksberg 2017). In the next paragraphs I am going to focus my dissertation on some specific neurodevelopmental disorders of monogenic origin and in particular Cyclin-Dependent Kinase-Like 5 (CDKL5) disorders, Creatine transporter deficiency syndrome, Krabbe disease, and Angelman syndrome. Recent studies indicate that common physiological mechanisms related to brain energetics might concur to the pathophysiology of these disorders, with upregulation of respiratory chain complexes, elevated ROS production, decrease in ATP production, increased synthesis of TCA cycle and glycolytic enzymes (Oyarzábal et al. 2021).

1.2 CDKL5 deficiency disorder

A recent review on CDKL5 disorder (Jakimiec et al. 2020) describe the pathology from a molecular/gene prospective to a clinical and therapeutic point of view. The human Cyclin-Dependent Kinase-Like 5 (*CDKL5*) gene (MIM 300203), located on the X-chromosome (Xp22.13), is composed of 24 exons (Diebold et al. 2014). *CDKL5* is highly expressed in the developing brain with implications in neuronal survival, development, and synaptic function (Fuchs et al. 2014; Olson et al. 2019). Despite the clear involvement of *CDKL5* for proper brain development, as evidenced from the available clinical and molecular data, *CDKL5* functions are still far from being understood (Kilstrup-Nielsen et al. 2012). The CDKL5 protein belongs to the CMGC family of serine/threonine kinases (including cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK), and CDK-like kinases). It is characterized by an N-terminal catalytic domain (amino acids 13–297), homologous to that of the other CDKL-family members and has roles in cell proliferation, neuronal migration, axonal outgrowth, dendritic morphogenesis, and synapse development and function in the adult brain (Zhu and Xiong 2019). *CDKL5* has been shown to regulate cell proliferation (Fuchs et al. 2014; Barbiero et al. 2017). In human neuroblastoma cells, CDKL5 is upregulated after retinoic acid (RA) - induced differentiation. Forced expression of CDKL5 in these cells appear in arrest in the G0/G1 phase of the cell cycle and inhibition of cell proliferation (Valli et al., 2012), implying that CDKL5 is a negative regulator of proliferation. This was confirmed by the finding that CDKL5 deficiency in mouse brain increases proliferation rate in dentate gyrus granule cells (Fuchs et al. 2014). However, the mechanisms underlying CDKL5 function in proliferation remains elusive.

It has been shown that in the rodent brain, downregulation of CDKL5 in neural progenitor cells by RNA interference (RNAi) results in a delay in the migration of layer II -III pyramidal neurons (Chen et al. 2004). A recent study found that CDKL5 interacts with IQGAP1, an important regulator of cell migration and polarity, and determine the association of IQGAP1 with its effectors Rac1 and microtubule plus end tracking protein CLIP170 (Barbiero et al., 2017). This finding suggests that defective microtubule dynamics contributes to the migration defects of CDKL5 -deficient neurons. Cultured neurons retain many key aspects of neuronal development in vivo including axon outgrowth, dendritic morphogenesis, and synapse formation (Banker, 1988). By using cultured rodent neurons as a model system, Nawaz et al found that CDKL5 plays a role in axon development (Nawaz et al., 2016). Whether CDKL5 can regulate axon development in vivo has not yet been studied and requires future investigation. The first evidence supporting the critical role of CDKL5 in neuronal development came from a study using RNAi to investigate the effect of CDKL5 knockdown on dendrite development (Chen et al. 2004). When CDKL5 was downregulated by RNAi in those neurons, neurite growth and dendritic arborization were severely impaired (Chen et al. 2004). Similar to what was found in cultured neurons, silencing CDKL5 in vivo by in utero electroporation resulted in a reduction in dendritic arborization in layer II -III pyramidal neurons (Chen et al. 2004). The dendrite phenotype revealed by RNAi was confirmed in CDKL5 knockout mouse lines. In one line, the total length of apical dendrites was significantly reduced in cortical and hippocampal CA1 pyramidal neurons (Amendola et al. 2014), and in dentate gyrus granule cells (Fuchs et al. 2014). The mechanism by which CDKL5 controls dendrite morphogenesis is not fully understood.

In dendritic spines, CDKL5 is highly enriched in the postsynaptic density (PSD), which suggests that this protein participates in synapse development and function. Della Sala et al., demonstrated that CDKL5 plays a crucial role in the organization and maintenance of synaptic structure *in vivo*. Indeed, new spines were normally generated but failed to stabilize and were eliminated at an abnormally high rate in the absence of CDKL5. This deficit resulted in a strong decrease in spine density of pyramidal cells, nearly half as much, that was accompanied by a decrease of PSD-95-positive puncta together with a reduction of PSD-95 expression in the PSD-enriched fraction of synaptosomes. Morphological and molecular alterations of synapses were reflected by functional impairments consisting of a reduction in the frequency of miniature EPSCs, possibly due to decreased synapse number, and defective LTP (Della Sala et al. 2014).

Even if the data regarding CDKL5 protein expression in different tissues are somewhat conflicting, the transcription data have been partially confirmed by western blotting using adult rat extracts (Chen et al. 2010; Lin et al. 2005). What is clear and commonly accepted is that, in the brain, CDKL5 levels reach the highest in conjunction with the development and differentiation of this organ. CDKL5 in fact is only weakly present during embryogenesis to get strongly induced in the early postnatal stages until P14, where after it declines (Rusconi et al. 2008)

Pathological variants in CDKL5 cause CDKL5 deficiency disorder (CDD, OMIM 300203, 300672), a developmental encephalopathy (DE) (Tao et al. 2004). Previously considered a “Rett variant,” this unique disorder (Fehr et al. 2013), has overlapping features with many of the developmental encephalopathies, disorders defined by genetic or presumed genetic etiology, severe seizures, and

intellectual or cognitive disability. Incidence varies from $\sim 1:40,000$ to $1:60,000$, (Kothur et al. 2018). Thus CDD is a diagnostic consideration in young children with severe, early-onset epilepsy.

Although most cases of CDD are the result of de novo mutations, cases of family history of CDKL5 mutations were also reported. This is probably the effect of germline mosaicism in one of the parents. Therefore, in these situations the offspring can inherit the mutated gene. Noteworthy is also the phenotypic divergence among siblings in whom the same mutation was confirmed. This suggests the involvement of epigenetic and environmental factors, as well as the relationship with the inactivation of the X chromosome, in determining the final phenotypic picture of particular patients (Jakimiec et al. 2020). Many of these de novo mutations result in both missense and nonsense mutations in the CDKL5 gene (Tao et al. 2004; Christiano et al. 2016). As most of the missense mutations occur in the region encoding the catalytic domain of CDKL5, it is believed that reduced activity of the enzyme specifically, its ability to modify amino acid residues is the direct cause of the onset of disease. Conversely, nonsense mutations in CDKL5 result in the formation of proteins lacking the C-terminus. The C-terminal region contains a sequence vital to the regulation of the intracellular localisation of CDKL5, and the mutations result in abnormal CDKL5 localisation patterns, as observed in an in vitro transient expression study (Fazzari et al. 2019).

Furthermore, nonsense mutations completely inhibit the ability of CDKL5 to interact with proteins that typically interact with its C-terminus. These observations illustrate the possible mechanisms of the involvement of nonsense mutations in disease onset. However, it is also possible that CDD is caused by the loss of CDKL5 expression if a premature stop codon triggers nonsense-mediated

decay (NMD) of CDKL5 mRNA. CDKL5 fragment was not detected in the R59X nonsense mutation knockin mice (Yennawar et al. 2019) indicating the occurrence of NMD.

1.2.1 Clinical Picture of the Disease

Primary symptoms of CDD include early-onset epilepsy (mostly drug-refractory), generalized hypotonia, psychomotor developmental disorders, intellectual disability, and cortical vision impairment. Moreover, a number of accompanying symptoms are reported, e.g., autistic features, poor social interactions, poor eye contact, hand stereotypy, vegetative disorders, gastrointestinal and orthopedic complaints, or dysmorphic facial features (N. Bahi-Buisson and Bienvenu 2012; Olson et al. 2019; Mazziotti et al. 2017; Lupori et al. 2019).

CDD is a X-linked disorder and affects female four times more often than men, suggesting that it is mostly a lethal mutation in male fetal life. The CDKL5 mutation is diagnosed in 8–16% of girls with early-onset epilepsy (Nadia Bahi-Buisson et al. 2008; N. Bahi-Buisson and Bienvenu 2012). However, in the population with early infantile epileptic encephalopathy (EEIE), this mutation was confirmed in 28% of girls and only in 5.4% of boys (Mei et al. 2014). The number of affected boys confirms the fact that the course of the disease is much more severe in boys, and often results in death in the first or second decade of life (Mirzaa et al. 2013). Inactivation of the X chromosome seems to be one of the most significant factors determining the severity of symptoms in female (Archer et al. 2006).

Phenotypically, the the pathology is heterogeneous not only in relation to gender. Case reports ranging from mild to severe forms of epileptic encephalopathy: mild forms, however, are significantly less prevalent. In those cases, patients

walk unaided, use simple languages, and epilepsy is controlled pharmacologically. Severe forms of epileptic encephalopathy include drug-resistant epilepsy, microcephaly, mental and psychomotor retardation, generalized hypotonia, and cortical visual impairments. Despite extensive studies in this respect, the correlation between the type or location of the mutation and the severity of symptoms cannot be clearly determined (Jakimiec et al. 2020).

Epileptic seizures are usually the first symptom of CDD. In 96.9% of patients, the first episodes are found in the first six months of life, and in the first three months of life in 90% (usually week 6) (Olson et al. 2019).

Early manifestation and diagnosis have a huge impact on the quality of life of patients and their families, as well as on the psychomotor and intellectual development. Epileptic episodes may have different morphology. Epileptic spasms usually occur at the onset of the disease in 23% of patients and at various stages of the disease in 81% of patients. Other possible types include absence, partial, myoclonic, tonic seizures, and limb spasms (Zhao et al. 2014).

Over time, seizures become generalized or multifocal with the most prevalent tonic-clonic manifestations. Recently, a new model of epileptic seizure evolution has been proposed as a result of the analyses of the morphology of seizures in children with CDD. Attention was paid to three phases: hypertonic (intense movements, muscle jerks), tonic, and epileptic. This seizure model was reported in 56% of subjects (N. Bahi-Buisson and Bienvenu 2012; Olson et al. 2019; Artuso et al. 2010).

Epilepsy in severe CDD shows high drug resistance (84%) (Frullanti et al. 2019). Frequent and intense epileptic episodes significantly affect the psychomotor and intellectual development of children, and result in epileptic encephalopathy, which has a secondary effect on the functioning and the quality of life of patients

caregivers (Amin et al. 2017).

Intellectual disability and delay in psychomotor development affect all patients with CDD. In almost all patients, intellectual disability ranging from moderate to more significant degree: patients achieve an IQ score of no more than 40 (Frullanti et al. 2019). The impairment seems to be mainly related to speech and small motor skills compared to achievements in gross motor skills (N. Bahi-Buisson and Bienvenu 2012).

Nearly 66% of girls with CDD can sit unsupported, 25% can stand, 21% can stand up from the sitting position, 23% of patients walk unaided until the age of 4.5 years, and 13% of patients can run at any stage of development. However, only about 35% of examined boys can sit unsupported. Isolated cases of affected boys who were able to stand (2/18; 11%) or walk independently (1/18; 5.5%) were reported (N. Bahi-Buisson and Bienvenu 2012; Fehr et al. 2016; Frullanti et al. 2019). About 50% of the girls achieve grasping ability up to five years of age and 13% of girls achieve pincer grip at any stage. Boys show poorer development of small motor skills, only 10% develop grasping ability up to two years of age, and only one boy achieved pincer grip at that time (Fehr et al. 2016).

Speech disorders in patients with the CDKL5 mutation are more severe than those related to gross motor skills. Studies indicate that 44% of patients babbled up to the age of six, and only 16% of patients were able to use single words up to seven years of age. In turn, only 7.5% of girls used simple sentences (in the literature, one case of a boy able to speak single words was also reported). Symbols, gestures, facial expressions, or vocalizations are the most common methods of communication used by about 65% of girls. In boys, only one in four communicated using gestures or vocalizations (Nadia Bahi-Buisson et al. 2008;

N. Bahi-Buisson and Bienvenu 2012; Fehr et al. 2016; Fehr et al. 2013; Frullanti et al. 2019).

Despite severe psychomotor retardation and significant intellectual disability, developmental regression is rarely observed in CDD patients. It affects approximately 20% of patients. This is the feature differentiating CDD from Rett syndrome, in which the loss of previously achieved milestones is a typical symptom. Eighty percent of patients with CDD present with retardation or inhibition of psychomotor development. Regression is reported in the cases of severe epileptic encephalopathy and is usually related to communication skills and manual dexterity (Fehr et al. 2016).

Generalized hypotonia affects all patients. However, it is more severe in boys (Fehr et al. 2016). Scoliosis is its relatively common orthopedic complication in children with CDD and occurs in 8.3% of patients. Rehabilitation, orthopedic corsets and surgical treatment are used to treat scoliosis (Olson et al. 2019; Frullanti et al. 2019).

Cortical visual disturbances are common and occur in 80% of patients. They manifest clinically as lack of fixation and lack of following objects with the eyes. Avoidance of eye contact is observed in 40.6% of patients, which is also interpreted as one of the autistic features. Less commonly reported symptoms include rotational or horizontal nystagmus (Nadia Bahi-Buisson et al. 2008; N. Bahi-Buisson and Bienvenu 2012; Mei et al. 2014; Artuso et al. 2010; Frullanti et al. 2019).

Hand stereotypies occur in 85.7% of patients and are one of the most common accompanying symptoms. Their prevalence is six-fold higher in girls and reaches 91% compared to 15% in boys. Putting hands in the mouth and clapping are most commonly observed. Symptoms usually appear in the first year of life and

are more intense over time (N. Bahi-Buisson and Bienvenu 2012; Frullanti et al. 2019). Crossing lower limbs is also common. Cases of episodic choreoathetosis, akathisia, and dystonia were also reported (Olson et al. 2019).

The head circumference at birth is normal in most patients (93.8%), but the inhibition of head circumference growth is observed in the first years of life. In 44.4% of patients with CDD this size indicates the result of <3 percentile as early as at the age of two (Frullanti et al. 2019). Vegetative disorders are a diverse group of symptoms associated with CDD: and include respiratory disorders (apnea or hyperventilation), gastrointestinal disorders, dilated pupils, facial flushing, or cooling of the limbs. These symptoms are almost three times more prevalent among the female gender (N. Bahi-Buisson and Bienvenu 2012; Olson et al. 2019; Frullanti et al. 2019).

Significant problems are observed in relation to nutrition and affect almost all patients (Frullanti et al. 2019): 79.3% are orally fed, of whom only 5.3% of patients are independent in this respect. At least one episode of choking per week during feeding is found in almost 75% of orally fed patients, which drastically increases the risk of aspiration and pneumonia. Due to feeding and swallowing disorders, 20.7% of patients are given enteral nutrition (gastrostomy or gastric tube). Of note, over 40% of patients show body weight below the 25th percentile (Frullanti et al. 2019).

Sleep disorders are common in approximately 86.5% of patients with CDD, of whom 58.5% present with somnambulism. However, the entire spectrum of sleep disorders is observed (problems with falling asleep, awakening, breathing disorders during sleep, difficulty waking up, or pathological drowsiness). The highest intensity of symptoms is observed in patients aged 5–10 years (Mangatt et al. 2016). Discreet dysmorphic features are observed in a small number of

patients (5.7%) and they mostly include deep set eyes, straight eyebrows, slightly short upturned nose, large ears, high forehead, or prominent lips (Olson et al. 2019; Archer et al. 2006). Other less commonly reported symptoms include bruxism (44%) and arrhythmias, hypersensitivity to touch, apraxia, or tetraplegia. It should be of note that CDD and many other syndromes characterized by frequent epileptic seizures may carry the risk of Sudden Unexpected Death in Epilepsy (SUDEP) (Olson et al. 2019; Frullanti et al. 2019; Amin et al. 2017).

1.2.2 Imaging Studies

Brain MRI images in the first months of life rarely show any abnormalities. Approximately 50% of 6-year-old children present with changes in neuroimaging. However, these are not very specific and are mostly related to (frontal) brain atrophy and white matter enhancement, mainly involving temporal lobes. Some patients also presented with wide sulci, widening of the ventricular system, or cerebellar atrophy. These changes are of different severity and occur in many other conditions. Therefore, they cannot be used as the basis for diagnosis, differentiation, or prognosis (Nadia Bahi-Buisson et al. 2008; Olson et al. 2019; Archer et al. 2006; Mei et al. 2014).

In a recent paper of Y. Tang et al., they first describe quantitative MRI morphological abnormalities in young children (3 years of age) with CDD. They detected global morphological deterioration in the brains of children with CDD, significant morphological abnormalities in 3-year-old CDD patients, which suggests that surface-based quantitative measurements might serve as potential sensitive markers for evaluating subtle structural abnormalities in these patients. The most striking volume loss found by Y. Tang et al. study was detected in the subcortical regions, moreover they found volume loss and cortical thinning in

the occipital, parietal, and temporal lobes (Y. Tang et al. 2021).

Finally, they longitudinally observed one patient, finding a progressive volume loss over time. The age-related morphological alterations in CDD patients also showed three different patterns varying across different brain regions, which might result from uneven regional disturbances by CDD, or regional heterogeneity of volumetric dynamics along with brain development, or a combination of these two factors (Y. Tang et al. 2021).

1.2.3 Therapy

Currently, there is no targeted therapy which could solve the underlying problem related to CDKL5 disorders. Therapeutic methods in patients with CDD are based on symptomatic drugs to control the most problematic complaints causing disability. Epilepsy is the major problem impacting the quality's life of patients and family. The choice of an antiepileptic drug should be based on the current seizure type and EEG recordings. However, resistance to antiepileptic drugs in CDD reaches 84%.

Data on the efficacy of seizure therapies is limited. A review of anti-seizure medication response in 39 individuals with CDD found a responder rate (defined as 50% seizure reduction) to at least one anti-seizure medication of 69% at 3 months, 45% at 6 months and falling to 24% at 12 months. Medications with the highest rates of seizure reduction at 3 months included felbamate, vigabatrin, clobazam, valproic acid, steroids, lamotrigine and zonisamide. The most commonly used anti-seizure medications in CDD were broad spectrum, including clobazam, valproate, topiramate, levetiracetam and vigabatrin and 29.6% of individuals were treated with steroids or ACTH. Infantile spasms in individuals with CDD are often refractory to first-line therapies. The ketogenic

diet has modest efficacy in treating epilepsy in CDD. The largest cohort reported 104 individuals with CDD treated with median ketogenic diet duration of 17 months and reductions in seizure frequency in 61/104 (58.7%), consistent with data from the Boston Children’s Hospital COE.^{16,66} Side effects of the ketogenic diet occurred in 31.7% of individuals. Palliative surgeries for refractory epilepsy include vagus nerve stimulation (VNS) and corpus callosotomy. Among 220 individuals with CDD with parent-entered data, 17% had a VNS implanted and 69% of parents reported reduced seizure frequency. In the International CDKL5 Disorder Database at least 7/10 individuals had some improvement in seizures following corpus callosotomy of whom two had a longer than 6 month period of seizure freedom (unpublished data)(Olson et al. 2019). Patients with CDD are currently being recruited to participate in one of four clinical trials: Ganaxolone, Ataluren, fenfluramine, and TAK-935/OV-935 (<https://clinicaltrials.gov>). These trials are motivated by an understanding of the biology of the disorders without prior pre-clinical studies in rodent models of CDD. Ganaxolone is a high-affinity allosteric modulator of GABAARs that acts to restore the balance of excitation and inhibition (Carter et al. 1997). The Ganaxolone/Marigold Study (Marinus) is a double-blind, placebo-controlled phase 3 trial that examines the effect of Ganaxolone on seizures, behavior, and sleep in patients between the ages of 2 and 21. These endpoints proved useful in previous non-CDD clinical studies with Ganaxolone. PTC Therapeutics, a NYU-based company, has initiated a phase 2 crossover study of Ataluren for the treatment of drug-resistant epilepsy in patients with nonsense mutations with CDD. There has been some concern that Ataluren does not effectively cross the blood–brain barrier; however, a proof of principle study indicated that at higher doses Ataluren breaches the blood–brain barrier in the infantile neuronal ceroid lipofucinosi mouse model,

where it increases the lysosomal enzyme palmitoyl-protein thioesterase 1 (PPT1) enzyme activity and protein levels (J. N. Miller et al. 2015). The fenfluramine ZX008 study (NYU, Zogenix) is an open-label trial of fenfluramine for treatment of seizures in patients with CDD. Fenfluramine was shown to be effective in open-label studies of Dravet syndrome and zebrafish model of Dravet syndrome (Schoonjans et al. 2017; Dinday and Baraban 2015) and is being considered for its utility in CDD. Fenfluramine has a novel mechanism of action that includes multiple receptors. It is shown to act both on the release and inhibition of serotonin reuptake as well as a positive allosteric modulator at the σ -1 receptor that is involved in modulation of glutamate (Rodríguez-Muñoz et al. 2018). The final agent TAK-935 (Takeda, Ovid) is a potent, highly selective inhibitor of the enzyme cholesterol 24-hydroxylase CH24H that is being used in the study of rare pediatric epilepsies. CH24H is predominantly expressed in the brain, where it plays a central role in cholesterol homeostasis. CH24H converts cholesterol to 24-S-hydroxycholesterol(24HC) which then exits the brain into the blood plasma circulation. CH24H is involved in over-activation of the glutamatergic pathway through modulation of the NMDA channel (Sodero et al. 2012; Paul et al. 2013), thus suggesting a potential role in epilepsy.

1.2.4 Modeling CTD Features in Animals

Although rare, CDKL5 syndrome is a major social issue, with patients demanding lifelong assistance, and the severity of the disease burdening families and the health-care system. Therefore, there is a pressing need to identify effective therapies for this disorder. Animal models are foundational resources for studying basic biological processes, disease pathogenesis and novel therapies. Over the past ten years, several models of CDKL5 syndrome have become available.

In the next sections, I will provide an overview of the different approaches used to generate CTD models, comparing cognitive, behavioral and functional phenotypes expressed by these animals.

CDKL5-Knockout mouse

Several CDKL5 knockout (KO) mouse models have been generated to understand how CDKL5 dysfunction leads to neurological defects in CDKL5 disorder (Wang et al. 2012; Amendola et al. 2014; Okuda et al. 2017; S. Tang et al. 2019). Despite the lack of overt epilepsy, CDKL5 KO mice exhibit numerous behavioral deficits across motor, sensory, cognitive, and socioemotional domains that are reminiscent of human symptomatology. These behavioral defects are associated with morphological alterations, such as reduced dendritic arborization of hippocampal and cortical neurons, deficits in dendritic spine density and organization, and altered connectivity (Wang et al. 2012; Amendola et al. 2014; Okuda et al. 2017).

In 2012, Wang et al. were the first group to report on the CDKL5-knockout mice, showing that loss of CDKL5 causes a characteristic clinical phenotype of CDD, including decreased fear sensitivity, impaired social behaviour, and decreased learning ability. Wang et al. reported that our CDKL5 knockout mice mirror the latter features, but not early-onset seizures. CDKL5 $-/y$ mice display hyperactivity, motor defects, reduced anxiety, decreased sociability, and impaired learning and memory. While video-EEG monitoring revealed an absence of spontaneous seizures in CDKL5 $-/y$ mice, ERP analysis showed attenuated and delayed ERP polarity peaks suggestive of impaired neuronal connectivity. These behavioral and electrophysiological impairments are unlikely the secondary effects of an epileptic neuronal network, suggesting they are consequences of

CDKL5 loss-of-function. Time-frequency analysis of ERPs identified a specific deficit in event-related δ , θ , and α low-frequency neuronal oscillations in CDKL5 $-/y$ mice. θ oscillations are proposed to support encoding and retrieval of memory, the impairment in event-related power and PLF at the θ frequency are consistent with the learning and memory deficits in CDKL5 $-/y$ mice and the prominent intellectual disability observed in patients with CDKL5-related disorders

Many analyses of CDKL5-knockout mice have been reported. Motor impairment and loss of learning and/or memory are most often reported, but behaviours similar to that seen in the attention deficit hyperactivity disorder and sleep apnoea syndrome have also been observed.

As conventional knockout mice, mouse models lacking exon 2, 4, or 6 have been established (Wang et al. 2012; Amendola et al. 2014; Okuda et al. 2017; Okuda et al. 2018). The CDKL5 mouse model of Amendola et al., recapitulate several core features of CDKL5 disorder and serve as a useful animal model of the disease. In particular, CDKL5 knockout mice showed hind-limb clasping, hypoactivity, defective head tracking, and abnormal EEG responses to convulsants, features that may model the stereotypic hand movements, hypotonia, eye-tracking abnormalities, and seizures, respectively, reported in the human condition. However, they failed to detect any spontaneous seizure or epileptiform activity in mutant mice on two different genetic backgrounds, interpreting these findings as an indication that CDKL5 impacts on the development of epilepsy in humans in a manner significantly different from mice. The phenotype of CDKL5 knockout mice partially overlaps with that of *Mecp2* knockouts. Both mutants showed hind-limb clasping, hypoactivity, and reduced dendritic branching of cortical neurons. Importantly, no evidence emerged for altered BDNF expression or function in CDKL5 knockout mice suggesting that these pathways

may not play a major role in common features of CDKL5 disorder and Rett Syndrome (Amendola et al. 2014). In Okuda et al, they generated the CDKL5 KO mice, and identified hyperexcitability in response to NMDA and postsynaptic overaccumulation of GluN2B-containing NMDARs in the hippocampus. The GluN2B-selective antagonist ifenprodil abrogated the NMDA-induced hyperexcitability of CDKL5 KO mice. These data indicate that CDKL5 plays an important role in controlling postsynaptic localization of the GluN2B-SAP102 complex in the hippocampus, and that aberrant NMDAR-mediated transmission underlies the pathological mechanisms of the CDKL5 LOF. These CDKL5 KO mice exhibit an aberrant and excessive accumulation of GluN2B at the PSD in the hippocampus (Okuda et al. 2017). Recently, R59X mutation knockin mice have also been developed (Yennawar et al. 2019). Hindlimb clasping, an indicator of cerebellar ataxia, was commonly increased in exon 4-deficient and R59X knockin mice compared with wild-type (Yennawar et al. 2019; Amendola et al. 2014). In addition, impairment of social behaviour by the three-chamber test is commonly observed in exon 6-deletion and R59X knockin mice (Yennawar et al. 2019; Wang et al. 2012). Interestingly, motor coordination deficiency by the rotarod test is common in exon 6-deficient mice and R59X mutant mice, but not in exon 2-deficient mice (Yennawar et al. 2019; Wang et al. 2012; Okuda et al. 2018). Moreover, mice lacking exon 4 have altered kainic acid sensitivity, whereas mice lacking exon 2 show no difference in kainic acid-induced epilepsy scores (Amendola et al. 2014; Okuda et al. 2017).

1.2.5 Preclinical testing of potential therapeutic strategies

Studies have been conducted to develop precise therapy based on the biological, metabolic and genetic basis of CDD. These studies include the use of

NMDA (N-methyl-D-aspartate) receptor modulators (S. Tang et al. 2019); allopregnanolone (a neurosteroid that restores normal microtubule morphology) (Barbiero et al. 2017); or tianeptine, which is an antidepressant affecting AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (Tramarin et al. 2018). Insulin-like growth factor IGF-1 activating the Akt/mTOR pathway (Della Sala et al. 2014) and is able to rescue S6 phosphorylation, spine deficits, and PSD-95 decrease also in CDKL5 KO mice. Synaptic PSD-95 expression was increased by IGF-1 also in CDKL5 mice, whereas spine density was not affected, suggesting that each synapse could have had more PSD-95 or more PSD-95 puncta were present on the same spine or along dendritic shafts. Importantly, IGF-1 improved spine density also in adult CDKL5^{2/y} mice when the synaptic deficit was established. Moreover, repeated imaging of the same dendrites before and after the IGF-1 treatment showed that the increased spine density induced by IGF-1 was still present 20 days after the end of the IGF-1 treatment, suggesting that the spines induced by IGF-1 are long-lasting (Della Sala et al. 2014). The achievements of modern technology also allow the development of methods of protein substitution therapy, which was described by Trazzi et al.; TAT-CDKL5 fusion proteins were used, which, crossing the barrier, reach the CNS while maintaining the CDKL5 protein activity. This treatment with TATk-CDKL5 fusion protein reduces sleep apnea occurrence and stereotyped behavior (hind-limb clasping) in CDKL5 KO mice (Trazzi et al. 2018).

1.3 Creatine transporter deficiency syndrome

Creatine (Cr) is commonly known as a nutritional supplement increasing muscle mass and performance (Wallimann et al. 2011). However, Cr function is not

limited to muscle fibers and this molecule is a crucial hub for energy metabolism in every cell of the body. Since the slow diffusion of ATP and ADP is insufficient to preserve cellular ATP homeostasis, the Cr/phosphoCr (PCr) system evolved as a temporal and spatial energy buffer (Ellington 2001). Cr kinase (CK) catalyzes the reversible conversion of Cr and ATP into PCr and ADP. PCr is a “metabolically inert” phosphagen serving as energy storage for immediate regeneration of ATP and as a shuttle of high-energy phosphates between mitochondrial sites of ATP production and cytosolic compartments of energy consumption (Wallimann et al. 2011). One important feature of this reaction is the presence of tissue- and cell-specific CK isoforms, showing well-defined subcellular locations: indeed, the Cr/PCr network relies on the differential compartmentalization of mitochondrial and cytosolic CKs that, respectively, store available cellular energy as PCr, maintaining the ADP/ATP ratio favorable for the activity of ATP synthase, and regenerate the cellular ATP pool. Cells with high-energy demand, including those of skeletal, smooth and cardiac muscle, brain and kidney, express elevated levels of CK (Saks et al. 2007). Furthermore, several studies have attributed to Cr a modulatory role in neurotransmission, since it is released in activity-dependent manner within organotypic cultures of rat brain (Almeida et al. 2006; Peral et al. 2010) and acts as competitive agonist of GABAA and NMDA receptors (De Deyn et al. 1991; Koga et al. 2005; Neu et al. 2002; Royes et al. 2008). Finally, Cr has been described as one of the main cellular osmolytes (Alfieri et al. 2006; Bothwell et al. 2001) and antioxidants (Lawler et al. 2002; Sestili et al. 2011).

In physiological conditions, Cr is obtained either from dietary sources, including meat, fish and dairy products (J. T. Brosnan and M. E. Brosnan 2007) or from endogenous synthesis (J. T. Brosnan and M. E. Brosnan 2007). The latter is

a two-step reaction involving the enzymes l-arginine:glycine amidinotransferase (AGAT) and S-adenosyl-l-methionine:N-guanidinoacetate methyltransferase (GAMT). AGAT converts glycine and arginine to guanidinoacetate (GAA) and ornithine. GAMT transfers a methyl group from S-adenosylmethionine (SAM) to GAA, yielding Cr and S-adenosyl homocysteine (Wyss and Kaddurah-Daouk 2000). This apparently simple pathway is complicated by the fact that most cells do not express both AGAT and GAMT enzymes, and the transport of the synthesis intermediate GAA between tissues is needed: in mammals, it has been shown that the kidney contains high amounts of AGAT leading to the production of GAA that is transported to the liver for being methylated to produce Cr. In contrast, the central nervous system houses its own Cr-synthesizing machinery (Defalco and Davies 1961; Wyss and Kaddurah-Daouk 2000), but differential levels of AGAT and GAMT have been detected in brain cells, suggesting a network of intercellular cooperation for Cr synthesis and metabolism (Braissant et al. 2001; Hanna-El-Daher and Braissant 2016).

Following endogenous synthesis or nutritional supply, Cr is released in the bloodstream to nourish all the body cells. Cr is a polar hydrophilic molecule intrinsically unable to cross the lipid membrane and a specific Na⁺/Cl⁻-dependent transporter (Cr transporter, CRT) is required for the cellular uptake of Cr (Joncquel-Chevalier Curt et al. 2015; Nash et al. 1994). CRT is encoded by the SLC6A8 gene, which is located on the long arm of X chromosome and has a coding sequence of 13 exons (Geeta S. Iyer et al. 1996; Nash et al. 1994). The predicted protein consists of 635 amino acids with a molecular mass of about 70.5 kDa (Guimbal and Kilimann 1993). This transporter is a member of a superfamily of proteins (called SLC6), including the transporters for the uptake of neurotransmitters (e.g., dopamine, GABA, serotonin) and

amino acids (e.g., glycine) (Chen et al. 2004). The SLC6 family display a common three-dimensional structure, with 12 transmembrane (TM) domains, an extracellular loop between TM3 and TM4 with N-glycosylation sites, and N- and C-termini facing the cytoplasmic side of the membrane (Dodd and Christie 2005; Jiddeke M. van de Kamp et al. 2014b).

CRT is tightly regulated by extracellular Cr levels, with high Cr reducing the uptake activity (Wyss and Kaddurah-Daouk 2000). Moreover, cellular energy depletion inhibits CRT via the AMPK-mTOR pathway (H. Li et al. 2010). CRT is widely expressed in different tissues (Geeta S. Iyer et al. 1996; Nash et al. 1994; Jiddeke M. van de Kamp et al. 2014b), including the brain, where it has been predominantly detected in cortical and subcortical regions involved in motor and sensory processing, learning and memory, and the control of affective behavior (Lowe et al. 2015; C. S. Mak et al. 2009; Nash et al. 1994). At the cellular level, CRT is expressed in neurons and oligodendrocytes, and is present in capillary endothelial cells forming the blood-brain barrier (BBB), whereas it has been detected only in lesser amounts in astrocytes (Braissant et al. 2001). Thus, Cr can enter the brain crossing the BBB, but the blood-brain transport of Cr appears relatively inefficient, at least in the mature age (Braissant et al. 2001; Perasso et al. 2003; Tachikawa and Hosoya 2011). Interestingly, the expression of SLC6A8 gene is remarkably prominent in fast-spiking parvalbumin inhibitory neurons (Saunders et al. 2018; Tasic et al. 2018).

Inborn errors of Cr metabolism refer to three different syndromes caused by mutations in AGAT, GAMT and CRT coding genes and mainly characterized by cerebral Cr deficiency (Carmen Fons and Campistol 2016; Jiddeke M. van de Kamp et al. 2014b). These genetic disorders share a common clinical picture with developmental delay/regression, intellectual disability (ID), severe disturbance

of expressive and cognitive speech, autistic-like features, behavioral and motor abnormalities, and seizures in most cases (Jiddeke M. van de Kamp et al. 2014b). While AGAT (AGAT-D) (Bianchi et al. 2000; Item et al. 2001) and GAMT deficiency (GAMT-D) (S. Stöckler et al. 1994) are autosomal recessive conditions affecting Cr biosynthesis, CRT deficiency (CTD) is an X-linked disorder impairing the cellular uptake of Cr (Cecil et al. 2001; G. S. Salomons et al. 2003). Cr supplementation leads to the attenuation of symptoms in AGAT-D and GAMT-D patients. More specifically, Cr monohydrate improves epilepsy and movement disorder in GAMT-D children, whereas smaller beneficial effects on ID have been reported (Khaikin et al. 2018; Stockler et al. 2007). By contrast, AGAT-D is associated with a milder phenotype and a better response to Cr treatment (Alessandrì et al. 2020; Battini et al. 2006; Battini et al. 2017). Unfortunately, the same is not true for patients with CTD and this pathology is still orphan of an effective therapy.

1.3.1 Inheritance pattern and clinical signs of CTD

The disruption of SLC6A8 gene leads to the loss-of-function of CRT protein and the clinical manifestation of CTD (OMIM #300352). The most common genetic alterations associated with CTD are missense mutations and one-amino acid deletions, but frameshift, nonsense, translation initiation site mutations, aberrant splicing and multi-exon deletions have been detected as well. One third of patients carry de novo mutations, but somatic mosaicism is present in several mothers. It has been suggested that missense variants are correlated to residual CRT activity and milder phenotype (J. M. van de Kamp et al. 2013), but one patient with a single nucleotide change displayed severe refractory epilepsy (Margherita Mancardi et al. 2007).

The main hallmarks of CTD include global developmental delay, ID, language and speech disturbances, autistic-like traits, behavioral abnormalities, seizures and movement disorders (J. M. van de Kamp et al. 2013; Jiddeke M. van de Kamp et al. 2014b). CTD was originally described in a 6-year-old American boy with a history of mild mental retardation, central hypotonia, delayed speech and language development, one episode of status epilepticus, multifocal EEG alterations, and hyperintense signal in the right posterior periventricular white matter at the MRI (Cecil et al. 2001; Gajja S. Salomons et al. 2001). Proton-magnetic resonance spectroscopy (MRS) revealed the absence of Cr/PCr signal in the brain. Moreover, Cr levels were increased in plasma and urine, with a normal profile for other metabolites (Cecil et al. 2001). Since the supplementation with Cr did not improve clinical condition or MRS signal (Cecil et al. 2001), it has been hypothesized a defect in the cellular uptake of Cr. Consistently, the sequence analysis of SLC6A8 gene highlighted a nonsense mutation, resulting in an unstable and/or not properly folded CRT protein that was inactive (Gajja S. Salomons et al. 2001).

Twenty years after this first portrait, several case reports providing a detailed description of neurological and neuropsychological symptoms of CTD have been published (Battini et al. 2006; Battini et al. 2017; Bruun et al. 2018; J. M. van de Kamp et al. 2013). Moreover, a collaborative study analyzed the phenotype of 101 patients worldwide, drawing an overview of the clinical spectrum of CTD and correlating phenotypic traits with biochemical, genetic and molecular data (J. M. van de Kamp et al. 2013). The authors remarked that the degree of ID in the CTD population varies from mild to severe, with IQ scores usually in the range between 20 and 69. Moreover, ID becomes more pronounced with age and adult patients have a more severe phenotype (J. M. van de Kamp et al.

2013). They also underlined the possibility that the clinical presentation of CTD might include an age-dependent progression of neurological and psychiatric problems (J. M. van de Kamp et al. 2013). One of the most invalidating features of CTD is the alteration of speech and language development. The inability to speak is fairly profound, with very poor communication often restricted to a handful of words and vocalizations in most patients. Language comprehension is commonly limited as well (Clark and Cecil 2015). Besides cognitive and linguistic problems, attention deficit, hyperactivity, autistic-like features with social anxiety and stereotypic behavior, impulsive behavior, aggressive behavior, self-injurious behavior and obsessive compulsive behavior were common clinical traits of CTD (J. M. van de Kamp et al. 2013). Seizures were reported in about 60% of patients. The most common types are generalised tonic-clonic seizure and simple or complex partial seizures with or without secondary generalisation, while absence or myoclonic seizures occurred in a few patients. Children often displayed febrile seizures. In most cases, however, seizures are sporadic and well-controlled with antiepileptic medications (Bruun et al. 2018; J. M. van de Kamp et al. 2013).

Finally, other neurological symptoms, including mild to moderate motor dysfunction, hypotonia, spasticity, coordination dysfunctions, dystonia or athetosis, wide-based gait and ataxia, were consistently described in CTD (Bruun et al. 2018; J. M. van de Kamp et al. 2013). At the structural level, brain MRI revealed abnormalities in about 70% of children, whereas being silent in other cases. Mildly delayed myelination, T2-hyperintensities, thin corpus callosum, enlarged ventricles/extracerebral spaces and cerebral/cerebellar atrophy have been observed (J. M. van de Kamp et al. 2013).

This portrait clearly denotes that CTD mainly affects brain development

and function. However, non-neurological symptoms, such as gastrointestinal problems (feeding difficulties, vomiting, constipation), bladder dysfunction, cardiomyopathy, ophthalmologic abnormalities, were present in patients as well (J. M. van de Kamp et al. 2013; Jiddeke M. van de Kamp et al. 2014b).

Being an X-linked condition, CTD mainly affects males, but heterozygous females can be symptomatic as well. Learning troubles and mild ID were present in heterozygous female family members and three index heterozygous girls have been clinically identified as patients showing ID, behavioral problems and epilepsy (Cheillan et al. 2012; Mercimek-Mahmutoglu et al. 2010). The variability of heterozygous phenotypes is mainly due to the random inactivation of X chromosome in women.

1.3.2 Diagnostic methods and prevalence

The diagnosis of CTD involves several steps. Starting from physical examination and neuropsychological profiling, the diagnostic mainstay is based on: urine analysis, brain MRS, genetic sequencing (Clark and Cecil 2015). The biochemical analysis of urine is performed to quantify the urinary Cr:creatinine (Crn) ratio, where Crn is the breakdown product of Cr metabolism (Jiddeke M. van de Kamp et al. 2014b; Wallimann et al. 2011). Previous reports established that a Cr:Crn ratio higher than 1.5 suggests a diagnosis of CTD in males (Nasrallah et al. 2010; G. S. Salomons et al. 2003).

The urinary Cr:Crn analysis is becoming a more widely available test for the metabolic screening of IDs, but Cr disorders can lead to subtle metabolic alterations, with false results regularly detected (Arias et al. 2007; Jiddeke M. van de Kamp et al. 2014b) MRS imaging is the gold standard for diagnosing CTD and other Cr syndromes (Clark and Cecil 2015). However, the high costs

of MRS and the need of sedation in patients with ID and behavioral disorders make this technique not accessible as a first level—screening test (Nasrallah et al. 2010; Arias et al. 2007). Finally, molecular sequencing of SLC6A8 gene and the assessment of Cr uptake in cultured patient fibroblasts are needed to conclusively validate CTD diagnosis (Clark and Cecil 2015).

Despite a precocious onset of symptoms, the average age of diagnosis of CTD ranges from 2 to 20 years of age (J. S. Miller et al. 2019). The identification of early clinical indicators, alerting pediatricians to seek a specialty referral and/or conduct a urine screen, might anticipate CTD diagnosis. In a recent retrospective study of parent reports and medical records, the authors observed that very early feeding or weight gain issues presenting together with delays in motor function milestones should encourage primary care providers to consider rare metabolic disorders (J. S. Miller et al. 2019). Further research will examine the natural history of CTD, providing novel tools helpful in the follow-up of this condition and possibly monitoring the efficacy of potential therapeutic intervention treatment.

Overall, CTD emerges as a relatively common cause of X-linked ID. According to several reports, the worldwide prevalence of CTD could be estimated between 0.25 and 1% in males with ID (Arias et al. 2007; Clark and Cecil 2015), while 0.024% of females were heterozygous carriers (DesRoches et al. 2015). The prevalence increases to 4.4% when focusing on mental retardation of unknown origin (Lion-François et al. 2006).

CTD patients are often misdiagnosed with cerebral palsy in early postnatal age and with autism spectrum disorder (ASD) in later development. A very recent study presented the case of a 6-year-old boy showing speech delay, lack of several social milestones, including shared attention, pointing, imaginative play

and name responding, attention deficit, hyperactivity, stereotypical movements, avoidant and restrictive eating habits, and excessive fluid intake. A single episode of generalized tonic–clonic seizure occurred at 3 years of age. This clinical picture initially led to the diagnosis of ASD by Diagnostic and Statistical Manual of Mental Disorders, 5th edition criteria (DSM 5), with accompanying language impairment and ID. MRS, however, detected a significantly decreased Cr peak, directing to diagnostic investigations for disorders of Cr metabolism. Molecular analysis, indeed, revealed a novel, frameshift variant of SLC6A8 gene, confirming a diagnosis of CTD (Yıldız et al. 2020). This paradigmatic case report underlines the significance of expanding newborn screening for metabolic disorders to Cr deficiency syndromes.

1.3.3 Therapeutic perspectives in CTD patients

In contrast to other Cr deficiency syndromes (Battini et al. 2002; Andreas Schulze et al. 2001; Sylvia Stöckler et al. 1996), the attempts to rescue Cr content in the CTD brain by exploiting nutritional supplementations have been of limited success (Joncquel-Chevalier Curt et al. 2015). Cr supplementation, alone or combined with arginine, glycine, and/or S–adenosylmethionine, failed to restore brain Cr content and to resolve clinical signs in the majority of patients with CTD (C. Fons et al. 2008; Schjelderup et al. 2021; Villar et al. 2012). Indeed, dietary strategies increased cerebral Cr and improved clinical parameters only in milder cases with residual brain Cr, and probable residual CRT protein function (Anna Chilosi et al. 2008; Annamaria Chilosi et al. 2012). Moreover, long–term administration of arginine and glycine might induce adverse effects (JUN and WENNMALM 1994; Schjelderup et al. 2021; Villar et al. 2012).

A recent work suggested that betaine supplementation exerts positive effects

on CTD phenotype and is well tolerated (Schjelderup et al. 2021). However, this is an open trial without a placebo control and limited to the observation of two patients; further studies in a larger number of subjects following a well-designed study protocol are required to establish the true therapeutic value of betaine.

Altogether, these data show that no satisfactory treatments are still available for CTD disorder. The mainstay of care is support for families, envisaging palliative therapeutic strategies for managing seizures and behavioral problems.

1.3.4 Modeling CTD Features in Animals

Although rare, CTD represents a major social issue, with patients demanding lifelong assistance, and the severity of the disease burdening families and the health-care system. Therefore, there is a pressing need to identify effective therapies for this disorder. Moreover, little is known about the causal chain of events leading from Cr depletion to the impairment of multiple key behavioral domains, while delineating how the metabolic failure in CTD might converge into brain function alterations is a prerequisite of paramount importance for the development and assessment of therapeutic strategies. Animal models are foundational resources for studying basic biological processes, disease pathogenesis and novel therapies. Over the past ten years, several models of CTD have become available. They can be classified into two main categories: whole-body and brain- (or cell-) specific lines. In the next sections, I will provide an overview of the different approaches used to generate CTD models, comparing cognitive, behavioral and functional phenotypes expressed by experimental animals.

Whole-body rodent models

Four whole-body CTD models have been developed so far. Two of them are knockout (KO) mice carrying the deletion of exons 2-4 (KO(2-4)) (Skelton et al. 2011) or 5-7 (KO(5-7)) (Baroncelli et al. 2014) in the SLC6A8 gene, resulting in the absence of a functional CRT protein. With a similar method, a third KO mouse has been obtained by targeted disruption of SLC6A8 gene through the insertion of a beta-galactosidase/LacZ reporter expression cassette between the exons 5 and 7 (Stockebrand et al. 2018). More recently, Duran-Trio and colleagues generated using CRISPR/Cas9 technology a rat knock-in (KI) strain carrying the Y389C mutation of SLC6A8, a tyrosine-to-cysteine missense variation detected in CTD patients (Betsalel et al. 2012), which results in the complete suppression of the transporter activity (Duran-Trio et al. 2021; J. M. van de Kamp et al. 2013). As a model for CTD, the KI line has the advantage of carrying one of the mutations responsible for the disease in the appropriate protein context, potentially providing a more faithful picture of the pathological situation.

The comparative review of published studies revealed that the most relevant features of CTD are consistently present across these animal models. First, brain Cr levels were invariably depleted, although a mismatch among measured concentrations stands out. The discrepancy in absolute values, ranging from the total absence of Cr (Skelton et al. 2011) to a reduction by 70-90% with respect to wild-type (WT) littermates (Baroncelli et al. 2014; Baroncelli et al. 2016a; Duran-Trio et al. 2021; Kenea C Udobi et al. 2019), however, is likely attributable to the use of detection methods with different sensitivities, such as colorimetric assays, gas or liquid chromatography coupled to mass spectrometry, reversed

phase HPLC and MRS. Cr decrease was also reported in several peripheral organs, including skeletal muscle, heart, kidney, testes and serum, while higher Cr was properly found in urine (Baroncelli et al. 2014; Baroncelli et al. 2016a; Duran-Trio et al. 2021; Skelton et al. 2011; Stockebrand et al. 2018; Wawro et al. 2021). It should be noted that residual Cr is present in most tissues. This may result from compensatory uptake via alternative transporters. Indeed, the paralogous CRT gene *SLC6A10* is expressed in testis and brain (Bayou et al. 2008; G S Iyer et al. 1996), the second putative creatine transporter encoded by *SLC16A12* gene is highly expressed in the kidney and retina with low levels of expression in the brain as well (Abplanalp et al. 2013), while Loike and colleagues reported that a fraction of Cr transport into cultured rat L6 muscle cells could occur via a sodium independent mechanism (Loike et al. 1988). Additionally, endogenous synthesis may contribute to maintaining Cr levels and a moderate rise of GAA levels was observed in various body districts of mutant animals, suggesting that AGAT activity is upregulated by the Cr decrease (Baroncelli et al. 2014; Duran-Trio et al. 2021; Russell et al. 2014). Since these compensatory mechanisms may represent possible therapeutic druggable targets, further studies will be needed to elucidate how tissues can partially preserve Cr levels despite the CRT loss-of-function.

Another distinctive general trait shared by all whole-body models was reduced weight (Baroncelli et al. 2014; Baroncelli et al. 2016a; Duran-Trio et al. 2021; Stockebrand et al. 2018; Skelton et al. 2011; Kenea C Udobi et al. 2019; Wawro et al. 2021). Although an alteration of physical features is not obvious in most CTD patients, low weight gain, slender build, decreased height and poor developed muscular mass have been described (J. S. Miller et al. 2019; Jiddeke M. van de Kamp et al. 2014b), thus corroborating the translational value of experimental

animal strains.

As mentioned above, the hallmark manifestation of CTD is ID. This aspect can be effectively assessed in rodents through a series of tests evaluating different types of memory and cognitive performance (Tanila 2018). Using the Y maze, object-recognition test (ORT), Morris water maze (MWM), and contextual and cued fear conditioning (FC), it has been demonstrated that human ID is well recapitulated by CTD models, as they exhibit deficits in most of these tasks. More specifically, both KO(2-4) and KO(5-7) mice showed an altered performance in ORT and MWM, revealing the impairment of declarative and spatial memory (Baroncelli et al. 2014; Baroncelli et al. 2016a; Skelton et al. 2011). In contrast, ORT was not affected in KI rats (Duran-Trio et al. 2021). This discrepancy might depend on the different impact of CRT loss in the behavioral physiology of the two species or variations in the experimental settings used for testing. In KO(2-4) mice, compromised associative memory was observed as well, with reduced freezing responses to cued and contextual fear (Skelton et al. 2011). Mild deficits in the MWM were also described in heterozygous females of KO(2-4) mouse line, showing no defects in ORT and FC (Hautman et al. 2014). On the other hand, working memory in the Y maze was damaged in both mutant mice and rats (Baroncelli et al., 2014, 2016; Duran-Trio et al., 2021). Interestingly, the Y maze test was found to be the single variable most informative for the discrimination between KO animals and WT littermates (Mazziotti et al., 2020). Ease of testing is a key factor in the assessment of complex phenotypes and the availability of a simple and reliable tool for CTD classification is considered highly valuable as potential first-line screening in preclinical studies of therapeutics. Since numerous non-cognitive factors may lead to impaired performance in memory tasks, it is worth noting that most

studies reported a lack of anxiety and stress-related behaviors in CTD animals (Abdulla, Pennington, et al., 2020; Baroncelli et al., 2014; Skelton et al., 2011), nor were there changes in the affective behavioral domain. Despite a shorter latency to immobility in the tail suspension test, KO(2-4) mice appeared to be rather resilient to induced depression (Abdulla et al. 2020a).

In contrast, the analysis of locomotor behavior tightly connected hyperactivity to KO(2-4) mice, while KO(5-7) animals were found to be less active at least during the dark phase (Baroncelli et al., 2014; Skelton et al., 2011). These conflicting results might be derived by the different experimental conditions: indeed, locomotor activity of KO(2-4) mice was assessed for one hour in a dedicated chamber, whereas KO(5-7) animals performed the test over 24 hours in the home-cage. The presence of a new, challenging environment as opposed to a familiar one could account for the observed differences. Accordingly, behavioral outputs in patients suffering from ADHD tend to depend on different contexts or situations (Burt et al. 2005; McLoughlin et al. 2011). Altogether, these observations suggest that a slight, novelty-induced hyperactivity might characterize the phenotype of KO mice. In the last 5 years, growing evidence highlights the relevance of Cr in establishing and maintaining proper cognition throughout different developmental phases of postnatal life.

Indeed, the time-dependent deletion of SLC6A8 gene using a drug-inducible Cre recombinase driven by the human ubiquitin C promoter leads to cognitive defects only when tamoxifen treatment was started in newborns (postnatal day 5, PND5), whereas no deficits emerged in animals with late depletion of Cr transport (Kenea C. Udobi et al., 2019). Accordingly, it has been shown that Cr synthesis and uptake are essential during embryonic development (Braissant et al. 2005), and embryonic central nervous system (CNS) depends predominantly on

external Cr supply, thus suggesting that CRT deficiency might induce irreversible damages already in utero (Olivier Braissant et al., 2011). On the other hand, a longitudinal analysis of KO(5-7) mice unveiled the effects of long-term Cr shortage, demonstrating that cognitive impairment progressively deteriorates throughout adulthood in these animals and is accompanied by signs of early brain senescence, including synaptic loss, microglial activation, reduced hippocampal neurogenesis and lipofuscin deposition (Baroncelli et al., 2016). Moreover, the lifespan of KO(2-4) mice was significantly shorter with respect to WT littermates (Wawro et al. 2021).

Since the clinical picture of CTD patients includes multiple traits linked to ASD, they also examined sociability and stereotyped movements in CTD models (Kalueff et al. 2016; Rothwell et al. 2014; Silverman et al. 2010). Of the two ASD domains, only stereotypies were straightforward in CTD models. A significant increase in the time spent grooming themselves was, in fact, reported in both KO(5-7) mice and KI rats (Baroncelli et al., 2016; Duran-Trio et al., 2021). Using rotarod motor learning as a proxy for acquired repetitive behaviors in mice, we similarly found that the deletion of 5-7 exons enhanced formation of repetitive motor routines (Baroncelli et al., 2016). Conversely, no defects in social interactions were observed, as both exon 2-4 and 5-7 KO mice and KI rats displayed normal social preference and social novelty (Baroncelli et al., 2016; Duran-Trio et al., 2021; Skelton et al., 2011). Finally, one of the more relevant pathological features of CTD is epilepsy, which occurs in approximately 60% of patients (Jiddeke M. van de Kamp et al., 2014). To date, this aspect was investigated in a single study, which reported the presence of spontaneous seizures and increased susceptibility to kainate-induced epilepsy in KO(5-7) mice (Mazziotti et al., 2020). Genetic neurodevelopmental disorders often include

epilepsy as part of their clinical portrait, but the concordance of seizure phenotype between human patients and their model counterparts has been rarely reported (Fallah and Eubanks 2020), thus highlighting the good face validity of CTD models.

Using EEG recordings and intrinsic optical signal imaging, the same study identified a set of quantitative biomarkers for monitoring brain function in CTD: indeed, specific alterations in theta, alpha, beta and gamma EEG frequency bands and a paradox increase in hemodynamic cortical response were found in KO(5-7) mice. The clinical relevance of EEG spectral changes was demonstrated by the comparable dysfunction in theta, alpha and, to a lesser extent, gamma bands reported in CTD patients (Mazziotti et al., 2020). Moreover, non-invasive techniques, such as functional near-infrared spectroscopy (fNIRS), will allow the study of cerebral blood flow in CTD patients (Lloyd-Fox et al. 2010). Despite the availability of reliable tools for CTD diagnosis, the biomarkers discovered in this work will have a fundamental impact in the research and clinical setting, as they will optimize the follow-up of patients and represent additional, informative tools to investigate the efficacy of potential therapeutic strategies in preclinical, and possibly clinical, studies.

Animal models offer the unique chance to study the pathological determinants of CTD to a cellular and molecular level. Despite no evidence of gross alterations in brain weight and anatomy (Baroncelli et al., 2016; Skelton et al., 2011; Wawro et al., 2021), a more subtle reorganizations of neural circuits, including altered levels of neurotransmitters like glutamate, GABA and serotonin (Abdulla, Pennington, et al., 2020; Duran-Trio et al., 2021; Skelton et al., 2011), reduced density of inhibitory synapses, dysregulated microglia activation, and the impairment of hippocampal neurogenesis (Baroncelli et al., 2016), suggested

that CTD pathogenesis might present a multicellular profile with a complex network of cell-autonomous and non-autonomous effects. A relatively unexplored role of Cr is that of regulator of the immune system, where it contributes to the fine tuning of both the innate and the adaptive response (Bredahl et al. 2021). Even though a detailed discussion of this matter goes beyond the scope of this review, animal models of CTD played a pivotal role in understanding some of the Cr mechanisms of action in this picture. For example, Ji et al. showed that Cr was essential to regulate macrophage polarization and CRT loss-of-function caused a pro-inflammatory phenotype of these cells, with low Cr levels boosting host defence upon infection (Ji et al., 2019). Although enhanced inflammatory responses may have positive outcomes in some cases, there is also the other side of the coin. As mentioned above, KO(5-7) mice exhibited a marked increase in activated microglia (Baroncelli et al., 2016), the resident macrophage population of the brain, and it is well-known that excessive microglial inflammation is implicated in many neurological diseases (L. Du et al. 2017). It is still not clear to what extent microglia cells express SLC6A8 gene (Olivier Braissant et al., 2010; Tasic et al., 2018) or if polarization in these cells is governed by the same factors operating in their peripheral counterparts (Hu 2020; Ransohoff 2016), but the study from Ji and colleagues opens up the possibility that neuroinflammation in CTD models might be due to a similar mechanism.

In search of possible druggable targets, it has been also shown that Cr deficiency profoundly disrupts body metabolism (Baroncelli et al. 2016a; Perna et al. 2016), with negative repercussion at subcellular level, such as an upregulation of proteins involved in energy homeostasis and mitochondrial activity, and a significant increase in oxidative stress (Baroncelli et al. 2016a; Perna et al. 2016; Giusti et al. 2019; Stockebrand et al. 2018). Although further studies will be

needed to better elucidate the molecular pathophysiology of CTD, I believe that antioxidant drugs and inflammation inhibitors might be valid therapeutic venues for CTD cure. Besides the neurological phenotype, the main alterations exhibited by whole-body CRT KO models occur at the level of the skeletal muscle, where reduced Cr (Baroncelli et al. 2016a; Stockebrand et al. 2013; Stockebrand et al. 2018; Russell et al. 2014) was associated with muscle fiber atrophy, vacuolar myopathy (Stockebrand et al. 2018; Wawro et al. 2021), and significant abnormalities in strength, endurance, motor coordination and learning (Baroncelli et al., 2016; Skelton et al., 2011; Stockebrand et al., 2018). These findings are in apparent contrast with the normal levels of Cr detected in the muscle of two CTD patients (deGrauw et al. 2003; Pyne-Geithman et al. 2004). However, hypotonia is the second most common comorbidity in CTD patients (Dunbar et al., 2014; Jiddeke M. van de Kamp et al., 2014) and a more comprehensive examination of muscle Cr levels in CTD patients is required prior to dismissing the muscle phenotype of CRT KO mice as inconsistent with the human data (Perna et al. 2016). The open debate concerning the extent of the muscular phenotype in CTD patients and the validity of the murine models in recapitulating this aspect, combined with the finding that extraneural dysfunctions might underlie behavioral deficits in other neurodevelopmental disorders (Depino, 2013; Sherwin et al., 2018), lead the CTD scientific community to investigate the phenotype of brain-specific animal models.

Brain- and cell-specific conditional mouse models

Two different brain-specific KO mouse models of CTD have been generated in the last few years (Baroncelli et al., 2016; Molinaro et al., 2019; K. C. Udobi et al., 2018). Using the Nestin promoter (Tronche et al. 1999) to drive Cre-recombinase

expression, Baroncelli et al. removed exons 5-7 (Nes-KO(5-7)) (Baroncelli et al., 2016; Molinaro et al., 2019) and Udobi et al. exons 2-4 (Nes-KO(2-4)) from the SLC6A8 gene in postmitotic neurons, glial cells and BBB endothelial cells of the CNS (Molinaro et al. 2019). Brain-specific CRT loss-of-function was confirmed by a significant decrease of Cr content in various brain regions and a parallel rise in the local synthesis of GAA. Peripheral tissues, including muscle, heart and kidney were not affected (Baroncelli et al., 2016; Molinaro et al., 2019).

Regular age-dependent body growth and normal forelimb force was also observed in these animals as a result of preserved Cr levels in the muscle. Only in the adult age (i.e., PND100), Nes-KO(5-7) mice started to show a slight decrease in body weight with respect to control littermates, suggesting a possible disturbance in the neural mechanisms regulating food intake and whole-body metabolism (Molinaro et al., 2019). Even though the size of weight reduction was relatively modest, future studies will be needed to investigate hypothalamic function in Nes-KO mice and to assess the possible correlation between the lower body mass and the hyperactivity recorded in these animals (Molinaro et al., 2019; K. C. Udobi et al., 2018).

There is general consensus that Nes-KO mice have a global impairment in cognitive functions ranging from procedural skills to declarative and spatial memory, as demonstrated by their altered performances in the Y maze, ORT, MWM, and contextual and cued FC (Molinaro et al., 2019; K. C. Udobi et al., 2018). In line with the results obtained on the whole-body strain, cognitive failure reflected an impairment of hippocampal neurogenesis in PND180 Nes-KO(5-7) mice, showing a lower density of Ki67-positive cells in the dentate gyrus (Baroncelli et al., 2016). The behavioral data, however, were not entirely consistent between the two studies. Nes-KO(2-4) animals showed deficits in

ORT and MWM already at PND60 (K. C. Udobi et al., 2018), whereas Nes-KO(5-7) mice manifested a late-onset (PND100) memory disruption followed by an age-dependent increase of cognitive frailty (Molinaro et al., 2019). This discrepancy could be explained by differences in the experimental setting, but also in the motivational drive of the animals: indeed, K C Udobi et al. reported that Nes-KO(2-4) mutants fail also in the cued version of the MWM, which is specifically designed to assess the mouse motivation to reach a visible platform.

Interestingly, alterations of cognitive performance in Nes-KO mice were not due to motor dysfunction or high levels of anxiety: motor abilities and muscle strength were totally preserved in these animals, and the behavior measured in the open field arena was in the range of normal values. Although locomotor activity was slightly increased in Nes-KO animals, it is unlikely that moderate hyperactivity could account for the observed deficits (Hautman et al., 2014; Molinaro et al., 2019; K. C. Udobi et al., 2018). On the other hand, hyperactivity and attention deficits are commonly described in CTD patients (Farr et al. 2020; Jiddeke M van de Kamp et al. 2014a). Since hyperactivity in ubiquitous KO mice is presumably masked by the motor problems due to peripheral Cr deficiency, the presence of this phenotype in conditional mice represents an original finding, making them a viable model to study certain aspects of this disorder.

In contrast to whole-body mutants, the endophenotype of Nes-KO animals did not include an increment of stereotyped movements and repetitive behaviors, suggesting that extraneural Cr might play a crucial role in the development of autistic-like traits (Molinaro et al., 2019). Moreover, studies of the incidence of spontaneous epilepsy and susceptibility to proconvulsant treatment are not yet available for conditional models.

Altogether, these studies demonstrate that brain Cr deficiency leads to

significant learning and memory deficits, providing two valuable models for investigating nervous-system specific mechanisms of CTD.

In search of the cellular basis of CTD pathophysiology, it was demonstrated that SLC6A8 deletion from forebrain excitatory neurons reproduced at least partially the neurological phenotype of CTD. The decrease of brain Cr content in CaMKII-KO(2-4) mice was mirrored by a broad-spectrum disruption of cognitive abilities in adult animals with no alteration of motor function (Kurosawa et al. 2012). Moreover, home-cage activity increased in CaMKII-KO(2-4) mutants, suggesting that glutamatergic neurotransmission is crucially involved in the expression of ADHD-like traits characterizing CTD. In contrast, the selective loss of SLC6A8 in dopaminergic neurons (DAT-KO(2-4) mice) lead to spontaneous hyperactivity, while sparing general motor and cognitive functions (Abdulla et al. 2020b). These data are in agreement with the major involvement of the dopaminergic system in ADHD (Leo and Gainetdinov 2013; Tripp and Wickens 2009).

According to the previous literature about other neurodevelopmental disorders (Al Dahhan et al. 2019; Crawley 2012), these results highlight that choosing the proper animal model is of paramount importance for the preclinical drug development pipeline. Although the conditional CRT KO mice did develop cognitive impairment in the adult age, they failed to recapitulate the early pathological phenotype and the autistic-like traits of CTD patients, indicating that they do not represent a comprehensive tool for the evaluation of potential therapeutics of translational value. Thus, whole-body mutants emerge as a preferable model of CTD. However, brain- and cell-specific mice might be meaningful for in-depth investigations of the pathogenetic mechanisms underlying neurological deficits induced by Cr deficiency and the role of Cr in the maturation

of different cell populations of the nervous system.

1.3.5 Preclinical testing of potential therapeutic strategies

Studies on Cr deficiencies due to mutations of biosynthetic enzymes have shown that the replenishment of brain Cr is effective in ameliorating the clinical manifestation of the disease (Battini et al. 2002; Schulze et al. 2001; Stöckler et al. 1996), proving the reversibility of the pathological effects caused by Cr deficit. Thus, in vitro systems and animal models have been extensively employed for preclinical assessment of different strategies aiming to reinstate brain Cr levels in CTD patients.

Cyclocreatine (cCr) is an artificial analog of Cr which can enter cells independently of CRT (Enrico et al. 2013; Gorshkov et al. 2019) and can be phospho/dephosphorylated by Cr kinase (Gorshkov et al. 2019; McLaughlin et al. 1972), mimicking the metabolic function of Cr. This compound has been proposed as a possible replacement for endogenous Cr. The outcome of this pharmacological approach has been first evaluated in brain-specific CRT conditional knockout mice, reporting a beneficial effect for learning and memory deficits (Kurosawa et al. 2012). These results prompted a more rigorous preclinical study exploring the therapeutic efficacy of a chronic oral daily treatment with cCr at three different dose levels in animals where the ubiquitous CRT loss aligns better with the genetics and early onset of CTD (Cacciante et al. 2020a). I found that the minimum efficacious dose (MED) of cCr for improving both the cognitive defects and the epileptic phenotype was 46 mg/kg, whereas 14 mg/kg was sufficient to ameliorate the autistic-like picture of stereotyped behaviors (Cacciante et al. 2020a). No detrimental repercussions of cCr administration have been observed, including no cCr-related deaths, alterations in brain morphology or

exacerbation of spontaneous seizures (Cacciante et al. 2020a). However, despite efficient kinetics of brain penetration, oral cCr treatment showed only a partial efficacy in reversing the pathological CTD phenotype, with moderate effects upon cognitive functions and against seizures (Cacciante et al. 2020a). This is likely ascribable to the inability of cCr to release free Cr (Wyss and Kaddurah-Daouk 2000) and to its low affinity for Cr kinase (Annesley and Walker 1977; McLaughlin et al. 1972), preventing cCr from restoring all natural Cr functions. In addition, high doses of cCr have been recently described to increase seizure incidence and brain vacuolation in Sprague-Dawley rats, possibly because of the accumulation of toxic metabolites (Kale et al. 2020; Mikati et al. 2004). Thus, the window between the “No Observed Adverse Effect Level” (NOAEL) and MED is narrower than the safety margins established by FDA and the development of this drug has been discontinued. A parallel research line focused on creatine esters as a possible tool for restoring cerebral Cr content. Intriguingly, these Cr derivatives are highly lipophilic and can passively diffuse inside the cells, (Lunardi et al. 2006). where they are processed by cellular esterases delivering free Cr (B. Du 1971). Creatine benzyl ester (CBE) has been shown to increase the intracellular Cr and PCr pool in mouse hippocampal slices. However, CBE is rapidly degraded to benzyl alcohol and Crn with possible toxic outcomes for brain cells (Lunardi et al. 2006). Creatine ethyl ester (CEE) appeared to enter fibroblasts obtained from CTD patients (Fons et al. 2010), but further studies revealed that the lipophilicity of this compound was not sufficient to cross the plasmatic membrane independently from the Cr transporter and that its half-life was very short in aqueous solution (Adriano et al. 2011). Despite the positive effects on synaptic transmission reported in wild-type hippocampal neurons (Adriano et al. 2011), CEE has poor chemical stability and is particularly

vulnerable to gastric environment and plasma esterases (Katseres et al. 2009). Accordingly, CEE administration in CTD patients did not change Cr level in brain MRS and did not induce a significant improvement in neuropsychological performance (Fons et al. 2010). More recently, screening of Cr fatty esters on cellular models revealed that the incorporation of these prodrugs in endothelial, astroglial, and neuronal cells depends on the length of the carbonyl chain. This study identified dodecyl creatine ester (DCE) as the compound showing the most interesting properties of brain penetrability and diffusion (Trotier-Faurion et al. 2013; Trotier-Faurion et al. 2015). DCE was reported to increase Cr levels in fibroblasts of CTD patients (Trotier-Faurion et al. 2013) and intranasal administration of DCE-loaded microemulsion enhanced ATP in the brain of CRT KO mice, leading to a better performance in declarative memory (Ullio-Gamboa et al. 2019). Another Cr ester with high lipophilic character, di-acetyl creatine ethyl ester (DAC), was shown to counteract the harmful effects of CRT pharmacological blockade in brain slices at both the biochemical and electrophysiological level (Enrico Adriano et al. 2018). An alternative strategy to overcome the impermeability of plasmatic membranes to hydrophilic elements is to couple Cr to aminoacids or other molecules, which have their own specific carrier (Burov et al. 2011). Examples of such compounds are Cr-Gly-OEt and Cr-NHEt, which were capable of increasing the cellular content of Cr in brain slices after the block of CRT (Garbati et al. 2014) but have never been tested *in vivo*. The same was true for Cr gluconate (Adriano et al. 2017).

Finally, it has been recently suggested that some of the mutations in SLC6A8 associated with CTD symptoms might engender folding defects in the transporter, altering the intracellular distribution and the function of the protein. The chemical chaperone 4-phenylbutyrate (4-PBA) restored surface expression as

well as substrate uptake for several folding-deficient CRT mutants, providing a promising rationale for the study of 4-PBA in animal models (El-Kasaby et al. 2019).

In summary, these data suggest that Cr esters endowed with proper delivery systems to reach the brain parenchyma would be a good drug candidate for CTD patients. Despite the proof-of-concept demonstration provided by Ullio-Gamboa et al., however, further studies on CTD animal models are mandatory to determine in vivo the efficacy and the therapeutic window of these drugs, and to assess their toxicological profile. Moreover, preclinical multicentre randomized controlled trials testing the compounds in different animal models of CTD will significantly strengthen the reliability of the results obtained, setting the foundation for the translational success (Katz et al. 2012).

1.4 Other neurodevelopmental disorders

1.4.1 Krabbe disease

Krabbe disease (KRB) (OMIM #245200) is an autosomal recessive lysosomal disorder affecting the white matter of the central and peripheral nervous systems. KRB is caused by homozygous or compound heterozygous mutation in the galactosylceramidase gene (GALC; 606890) on chromosome 14q31. This results in a lysosomal storage disorder in which the gene for the enzyme galactosylceramidase is rendered defective through mutation (K. Suzuki and Y. Suzuki 1970), that produces the inadequate digestion of galactosylceramide, a main lipid component of myelin, and psychosine, a byproduct that can accumulate over 100-fold in the brain (Svennerholm et al. 1980). Psychosine is thought

to promote toxicity, resulting in extensive demyelination (Miyatake and Kuni-hiko Suzuki 1972; Tanaka et al. 1989; K. Suzuki and Y. Suzuki 1970). The massive loss of oligodendrocytes/myelin is thought to account for the absence of galactosylceramide accumulation in KRB (K. Suzuki and Y. Suzuki 1970).

KRB is also known as globoid cell leukodystrophy because of the presence of globoid cells (swollen microglia/macrophages filled with lipids, stained by Periodic acid–Schiff, and often multinucleated) in the CNS together with extensive demyelination (D’agostino et al. 1963; Malone et al. 1975).

Most patients present within the first 6 months of life with ‘infantile’ or ‘classic’ disease manifest as extreme irritability, spasticity, and developmental delay (Wenger et al. 2000). There is severe motor and mental deterioration, leading to decerebration and death by age 2 years. Approximately 10 to 15% of patients have a later onset, commonly differentiated as late–infantile (6 months to 3 years), juvenile (3 to 8 years), and even adult–onset forms. The later–onset forms have less disease severity and slower progression. These later–onset patients can be clinically normal until weakness, vision loss and intellectual regression become evident; those with adult onset may have spastic paraparesis as the only symptom. Disease severity is variable, even within families (Tappino et al. 2010). Late infantile and juvenile patients regress at an unpredictable rate but all will become severely incapacitated and die 2–7 years after diagnosis (Crome et al. 1973; Lyon et al. 1991). Some adolescent and adult patients have symptoms confined to weakness without intellectual deterioration, while others become bedridden and continue to deteriorate mentally and physically (Wenger et al. 2000; P. K. Thomas et al. 1984; Kolodny et al. 1991). Later–onset patients are a challenge for the clinician to suspect this diagnosis and then request a definitive test by a laboratory experienced in the measurement of GALC activity

(Wenger et al. 2000). Krabbe's disease is a genetic disorder, and its frequency varies widely with the population. It is a rare lysosomal storage disorder and the incidence in Europe is estimated to be 1 per 100,000 live births. A U.S. estimate of 1 per 250,000 was made after analyzing death certificates, but a more precise estimate can approach the European incidence (Barczykowski et al. 2012).

Imaging Studies and treatment approaches

Imaging studies conducted with computed tomographic scan and magnetic resonance imaging identified some useful aspects of this pathology: initially, some areas resulted in a symmetrical hyperdense signal that became hypodense during the course of the disease (Sakai 2009).

Currently, the therapeutic options are limited to asymptomatic or minimally symptomatic patients with Krabbe disease's infantile form. Some treatments are available and includes, hematopoietic stem cell transplant (HSCT) from a healthy donor and cord blood stem cell transfusion. HSCT helps the patient to populate the brain with microglia with normal function and good GALC enzyme activity, studies have shown its efficacy in halting the cognitive and motor decline in the patient. Instead, cord blood stem cells collected from an unrelated donor's umbilical cord are transfused into the patient. However, this procedure has also been beneficial to only those patients treated before symptoms appear (Sakai 2009).

Mouse models and preclinical tests on Krabbe disease

The twitcher, a neurological mouse mutant originally discovered at the Jackson Laboratory (Bar Harbor, Maine) in 1976, is an enzymatically authentic model of one of the classical human genetic leukodystrophies, Krabbe Disease or globoid

cell leukodystrophy (GLD). Since the first clinico– pathological documentation and the identification of the genetic defect of a lysosomal hydrolase, galactosylceramidase, it has been widely used as a convenient and useful experimental tool for investigations of the pathogenesis of and therapeutic approaches for GLD (Kunihiko Suzuki and Kinuko Suzuki 1995). The clinical phenotype of the twitcher mutant is transmitted as an autosomal recessive trait. Homozygous twitcher mice (*twi/twi*) are indistinguishable at birth from their unaffected littermates. Affected mice become generally less active than their littermates by 15-20 days. Early clinical signs include failure to gain weight and generalized tremulousness. At the terminal stage around 40 days, their body weight is about one-third of normal mice. Progressive muscle weakness and eventual paralysis develop, particularly conspicuous in the hind limbs and the neck muscles. Motor functions generally deteriorate rapidly after 20 days (Kunihiko Suzuki and Kinuko Suzuki 1995).

The brains of affected mice are slightly smaller than that of normal mice at the terminal stage but no other gross abnormalities are present. The characteristic pathology in the CNS mice is demyelination, accompanied by degeneration of oligodendrocytes and infiltration of macrophages, containing periodic acid-Schiff (PAS)-positive materials. The macrophages are singularly scattered or clustered around blood vessels. These pathological features are essentially identical with those of human Krabbe disease. Crystalloid or slender tubular inclusions, typical in human GLD, are present largely in the macrophages and the oligodendrocytes. The cerebral cortex and other regions of gray matter are relatively unaffected pathologically, except for occasional perivascular or perineuronal microglial cells containing similar inclusions. Unlike in the central nervous system, peripheral nerves are grossly abnormal. They are abnormally thick, translucent and firm and

can easily be distinguished from normal nerves by visual inspection. Although the pathology is largely confined to the nervous system, abnormal inclusions are frequently present in the renal epithelial cell (Kunihiko Suzuki and Kinuko Suzuki 1995).

Another murine model, the trs mouse, was developed using homologous recombination (Luzi et al. 2001). These mice contain the variant H168C on a FVB/N background, resulting in an 80-90% reduction in GALC activity (Luzi et al., 2001). Trs mice have increased levels of psychosine in the brain, however, the rate of accumulation is slower than that observed in Twitcher mice (Luzi et al., 2001). At PND 49, these mice display demyelination in both the CNS and PNS, and though there is macrophage infiltration, it is not as severe as that seen in the Twitcher model (Luzi et al., 2001).

Trs mice homozygous for the mutation are indistinguishable from their heterozygous and wild-type littermates for the first 25-30 days, but begin to show obvious signs of tremors and hind-limb weakness at this point (Luzi et al., 2001). These mice have an average lifespan of 50 days, but there is considerable variability, with the lifespan ranging from 40-63 days (Luzi et al., 2001). Due to the higher levels of GALC activity, later-onset of disease, and more variable lifespan, trs mice may be a more accurate model of juvenile-onset rather than infantile-onset GLD.

Preclinical testing in twitcher mice includes gene therapy, Rafi et al. demonstrated the potential of utilizing multiple CNS injections to optimize delivery and overall cross-correction. By injecting neonate mice intracerebroventricularly, intracerebellarly, and intravenously with rAAVrh10-GALC, high enzyme activity was achieved in the brain and cerebellum, and moderate to high activity was detected in the spinal cord and the sciatic nerve. In addition, treated mice

successfully lived up to 8 months and gained the ability to mate and care for newborns, among other phenotypic corrections.

Recently, Rafi et al. treated Twitcher mice on PND10-12 with a single tail vein injection of AAV2/rh10-mGALC. Treated mice survived significantly longer than untreated animals, and had a reduced kyphosis and tremors (Rafi et al. 2015). Tissues from treated animals had GALC activity at or exceeding WT levels in brain, spinal cord, and sciatic nerve, when measured at PND45-64 (Rafi et al. 2015). It is likely that the efficacy observed in this study is due to partial correction of both the peripheral and central nervous systems.

Enzyme replacement therapy (ERT) takes advantage of M6P receptor-mediated transport, in which M6P modified recombinant enzymes can be endocytosed by enzyme-deficient cells expressing the M6P receptor on their surface. This strategy will provide a transient source of active GALC to deficient cells and tissues, and has been evaluated in the Twitcher model. Weekly intraperitoneal (IP) injections of recombinant GALC were able to extend the lifespan of Twitcher mice to 47 days (Lee et al. 2005). Interestingly, the increase in lifespan was observed whether treatment started at PND 10 or at PND 20 (Lee et al., 2005). It is unlikely that systemic ERT through an IV or IP route will provide significant long-term efficacy since GALC does not cross the blood brain barrier. An alternative way to compensate for the low GALC activity is to inhibit the production of the cytotoxic molecule, psychosine. This approach is referred to as substrate reduction therapy (SRT) and has shown promise in other lysosomal storage diseases (Parenti et al. 2015). Although SRT is a promising approach, it will only slow the progression of disease. Because there is no current inhibitor for ceramide galactosyltransferase (CGT), the enzyme directly responsible for psychosine synthesis, enzymes further upstream must be targeted (LeVine et al.

2000). Due to the high oxidative stress seen in cells exposed to psychosine, antioxidant treatments have also been explored. Paintlia et al. hypothesized that psychosine accumulation could be altering vitamin D homeostasis, contributing to the demyelination seen in GLD patients and animal models. Therefore, heterozygous mothers and weaned Twitcher mice were fed a high vitamin D diet for the duration of their lives. The high vitamin D diet significantly extended lifespan to a mean of 50 days, and onset of tremor was delayed to PND 35 days (Paintlia et al., 2015). Treated mice had a slower rate of psychosine accumulation and reduced levels of demyelination compared to untreated animals (Paintlia et al., 2015).

1.4.2 Angelman syndrome

Angelman syndrome (AS) (OMIM #105830) is a neurodevelopmental disorder characterized by mental retardation, movement or balance disorder, typical abnormal behaviors, and severe limitations in speech and language. Approximately 70% of AS cases result from de novo maternal deletions involving chromosome 15q11.2–q13; approximately 2% result from paternal uniparental disomy of 15q11.2–q13; and 2 to 3% result from imprinting defects. A subset of the remaining 25% are caused by mutations in the gene encoding the ubiquitin–protein ligase E3A gene (UBE3A; 601623) (Kishino et al. 1997). In 1965, the English physician Harry Angelman described 3 patients who presented with a stiff, jerky gait, absence of speech, excessive laughter, and seizures. The disorder that came to bear his name is now recognized to affect approximately 1 in 15,000 individuals and is characterized by motor dysfunction, severe intellectual disability, speech impairment, seizures, hyperactivity, and autism spectrum disorder (ASD) as a common comorbidity (Williams et al. 2006).

Developmental delay in individuals with AS is usually observed within the first year of life. Though most individuals lack speech entirely, some who are mildly affected can acquire a few words. Receptive language is less impaired. Seizures occur in >80 % of patients, and onset is usually before the age of 3 years. Movement disorders include tremors, jerkiness, and ataxia. The characteristic behaviors of AS include mouthing of objects, happy demeanor with easily provoked laughter, attraction to water, hyperactivity, short attention span, and decreased sleeping. These features of AS can be seen in other neurodevelopmental disorders, leading to a broad differential diagnosis. Overlapping clinical features may indicate common neurophysiological pathways, most of which remain to be elucidated. The diagnosis of AS should be confirmed molecularly, in order to provide accurate information regarding prognosis, complications, and risk of recurrence. Precision in diagnosis helps to inform future research endeavors by establishing the genotype–phenotype relationship more clearly and providing a foundation upon which the success of new therapies can be gauged (Margolis et al. 2015).

Novel therapeutic approaches

The management of Angelman syndrome is mainly symptomatic as there is no curative treatment till now. However, since all patients with AS have one functional but silenced copy of paternal UBE3A, many attempts have been made to unsilenced the paternal UBE3A allele. The paternal UBE3A allele is silenced due to the SNHG14 transcript, which caused transcriptional interference. Then, one of the ways to activate the paternal allele would be to prevent the formation of the SNHG14 transcript. This has been successful in mice with topotecan, which is a topoisomerase inhibitor (Powell et al. 2013).

Antisense oligonucleotides (ASO) are also very promising. They function via RNA interference against SNHG14, causing its degradation (Meng et al. 2015a).

Mouse model and preclinical studies

Mouse models generated by targeted inactivation of Ube3a provide additional support to the causative role of UBE3A mutations in AS (Y. h. Jiang et al. 1998; Miura et al. 2002). Upon inheritance of Ube3a deletion through the maternal but not the paternal germline, mice recapitulate many features of the human disorder, displaying impaired motor function, seizures, and deficits in context-dependent and spatial learning. The observation that paternal imprinting of Ube3a occurs in mature neurons, but not in immature neurons, glia or non-nervous system tissues, reinforces the idea that the loss of UBE3A function in the central nervous system underlies AS pathology (U. Albrecht et al. 1997; Judson et al. 2014; Y. h. Jiang et al. 1998; Miura et al. 2002). Deficits in cognitive function of Ube3a, have been shown several times using fear conditioning and water maze learning paradigms (Y. h. Jiang et al. 1998; Van Woerden et al. 2007; Huang et al. 2013). But surprisingly, the learning deficits observed on these tasks tend to be mild, have not yielded consistent results across labs, and should therefore be interpreted with care (Born et al. 2017; Y. H. Jiang et al. 1998) as discussed by (Sonzogni et al. 2018; Silva-Santos et al. 2015). To model at least part of these behavioural dysfunctions, a series of behavioural tests are now used to test AS mice (Sonzogni et al. 2018). Open field assays (Prut and Belzung 2003; Seibenhener and Wooten 2015) and the elevated plus maze tests (Pellow et al. 1985; Walf and Frye 2007) have been extensively used to test the levels of anxiety in rodents. AS mice show stronger anxiety than their wild type controls when placed in the open field maze (Huang et al. 2013; Born et al. 2017). AS mouse

models consistently show changes in the marble burying paradigm (Born et al. 2017; Silva-Santos et al. 2015; Sonzogni et al. 2018), this test assesses rodent behaviour related to natural digging and burrowing behaviour (Gyertyán 1995; A. Thomas et al. 2009) , however, whereas many mouse models for ASD show increased burying behaviour, AS mice actually show reduced burying behaviour. Finally, AS mice show a reduced capacity for nest building (Sonzogni et al. 2018; Silva-Santos et al. 2015), but the precise translational value of this observation is unclear as it is unknown which brain areas are underlying this deficit.

Preclinical studies in mice model of AS includes intrahippocampal injection of AAV9/UBE3A partially rescued learning and long-term potentiation in the AS mice but showed no effect on motor symptoms (Daily et al. 2011). In a Cre-Lox inducible UBE3A mouse model, motor symptoms, long-term potentiation, and seizures were improved by reactivation of the gene in the newborn period, but behavioral symptoms were unchanged (Silva-Santos et al. 2015; Rotaru et al. 2020), argued to be due to only partially restored expression levels (Silva-Santos et al. 2015)(Silva-Santos et al. 2015). One intriguing approach is un-silencing the functional but dormant paternal copy in the CNS (Buiting et al. 2016), which was first shown to be possible by intracerebroventricular administration of the topoisomerase inhibitor topotecan, although off-target effects and overall toxicity would likely prevent the drug from being directly used (Buiting et al. 2016; Huang et al. 2011). Another promising approach is to activate the dormant paternal copy using an antisense oligonucleotide which resulted in partial rescue of UBE3A expression for 4 months and improvement in memory (cognitive) but no other domain symptoms in a preclinical study (Meng et al. 2013; Meng et al. 2015b). GTX-102, an antisense oligonucleotide based on the aforementioned mechanism, is currently in clinical trial stage. Recently, another group has demonstrated

that it is possible to use an AAV-delivered CRISPR Cas9 gene editing system to activate the paternal copy, partially restoring UBE3A levels and improving behavior phenotype in an AS mouse model (Wolter et al. 2020). The challenges to be addressed in developing an effective gene therapy for AS stem mainly from the genetic complexity with different subtypes, possible involvement of multiple genes in the most common and most severe subtype (maternal copy deletion) and the perhaps narrow physiologic expression range of UBE3A (Elgersma 2015), while the existence of the silenced paternal copy of the gene provides unique avenues for exploration.

Chapter 2

Aim of the study

Neurodevelopmental disorders are a heterogeneous group of pathologies affecting children in the first years of life. These conditions have a devastating impact on affected's children and their family's quality of life. There is no effective treatment for these diseases; pharmacological medications only include palliative and symptomatic drugs that are able, in mild cases, to partially relieve the most impacting symptoms like seizures, gastrointestinal and respiratory problems.

The efficacy study of novel potential treatments is, at least partially, hindered by the scarcity of unbiased, quantitative biomarkers for monitoring brain function. This is an important problem, because the phenotypic observation of behavioral endpoints might be highly prone to subjective bias, and the use of objective readouts for preclinical and clinical trials is crucial to properly evaluate the therapeutic response to new drugs.

The objective of this thesis was to take advantage of intrinsic spectral properties of oxygenated and deoxygenated hemoglobin to devise non-invasive biomark-

ers of brain function in different murine models of neurodevelopmental disorders, such as *cdkl5* deficiency disorder, creatine transporter deficiency syndrome, Krabbe disease and Angelman syndrome. Animal models for these disorders are available and recapitulate different critical features of human conditions, including learning and memory impairments, motor stereotypies, and visual deficits. Thus, they represent a very good model for studying pathogenetic mechanisms and therapeutic approaches. Since disruption of brain energy metabolism is a major disease mechanism linking these disorders, we hypothesized that the assessment of metabolic consumption might represent a sensitive readout for the analysis of possible alterations of brain function. Optical imaging techniques, such as imaging of intrinsic optical signal (IOS) and functional near-infrared spectroscopy (fNIRS), allow quantifying oxygen consumption in different regions of the cerebral cortex, providing an indirect measure of neuronal activity (Zepeda et al., 202004). In this framework, the analysis of visual phenotype is a paradigmatic model to evaluate cortical processing in different neurodevelopmental conditions (CIT). Thus, I used intrinsic optical signal imaging, to monitor visual amplitude in different mice models of neurodevelopmental disease.

On the other hand, behavioral analysis of mutant mice and drug-related outcomes represents an important goal in neuroscience, and many tests are available to meet this need. Nevertheless, classical paradigms used in neuroscience are subjected to different biases that reduced the reliability and accuracy of these measures (Gerlai 2001; Tanila 2018).

To minimize researcher bias, we have developed an entirely automated 3D printable chamber for appetitive conditioning tests based on operant conditioning (OC).

OC is a classical paradigm and a standard technique used in experimental psy-

chology in which animals learn to act to achieve a reward. With the application of this paradigm, it is possible to extract learning curves and measure reaction times (RTs). Both IOS and behavioral measurements are proxies of cognitive capabilities and can be used to evaluate the effectiveness of therapeutic interventions in mouse models of disease (Mazziotti et al. 2020a). Despite the availability of reliable tools for the diagnosis of these disorders, including biochemical and genetic analyses (Ghirardini et al. 2021; R. Hagerman and P. Hagerman 2021), indeed, the biomarkers discovered here will have a fundamental impact in the research and clinical setting at multiple levels: (i) their evaluation will allow clinicians to optimize the follow-up of patients, recognizing possible alterations of brain circuits during the progression of the disorder; (ii) the combination of behavioral and imaging testing provides a very reliable protocol to longitudinally monitor the efficacy of potential therapeutic strategies in preclinical, and possibly clinical, studies.

Chapter 3

Materials and Methods

3.1 Animals

Animals were maintained at 22°C under a 12-h light–dark cycle (average illumination levels of 1.2 cd/m²) and housed in standard cages according to current regulations about animal welfare. Food (4RF25 GLP Certificate, Mucedola) and water were available ad libitum. Genomic DNA was isolated from mouse tail (Dneasy Blood and Tissue Kit, Qiagen, USA) and genotyping was performed as previously described in Baroncelli et al.

CDKL5 mice The mice used for physiological characterization derive from the CDKL5 null strain in C57BL/6N background developed in (Amendola et al. 2014) and backcrossed in C57BL/6J for three generations. Male wt mice were bred with female hets to obtain mutant and wt littermates. Weaning was performed at P21–23. Colony founders were selected for the absence of the rd8 retinal degeneration allele spontaneously present in C57BL/6N mice.

To generate conditional knock-out mice lacking CDKL5 only from pallial structures, sparing most of the subcortical regions, we crossed homozygous mice bearing a floxed version of CDKL5, described in (Amendola et al. 2014), with mice expressing CRE recombinase under the control of *emx1* promoter (The Jackson Laboratory; B6.129S2-*Emx1*^{tm1}(cre)K^{rj}/J, stock no. 005628) (Gorski et al. 2002).

CTD mice I used male mice hemizygous for CrT exons 5-7 deletion (CrT-/y or knock-out (KO)) on a C57Bl6J background (Baroncelli et al. 2014), and their wild-type (WT; CrT+/y) littermates. Animals in each experimental group came from different litters, with a minimum of three litters to prevent possible litter effects.

For experiment with conditional KO: CrT+/y, nes-CrT+/y and nes-CrT-/y mice on a C57BL/6J background were generated as reported previously (Baroncelli et al. 2014). CrT+/fl females were crossed with nestin::Cre male mice (François Tronche et al. 1999). Animals with four genotypes were tested: wild-type animals (CrT+/y), mice expressing Cre-recombinase but not carrying the floxed allele (nes-CrT+/y), mice carrying the brain specific deletion of CrT (nes-CrT-/y) and mice expressing the floxed allele but not Cre-recombinase (CrTfl/y). PV-CrT mice on a C57BL/6J background were generated, crossing CrT+/fl females with Pvalb-IRES-Cre (Silvia Arber, <https://www.jax.org/strain/017320>), I tested mice carrying the Pvalb specific deletion of CrT (PV-CrT -/y) compared to PV-CrT +/y.

Twitcher mice The original breeder pairs (heterozygote), on the C57BL/6 background, were obtained from Jackson Laboratory (Bar Harbor, ME)

<https://www.jax.org/strain/000845>) The mice used for physiological experiment were heterozygous mice (C57BL/6J, twi/+) and wt littermate.

UBE3A mice Ube3a maternal deletion mice (AS model) were generated by Jiang and colleagues Jiang1998-yj. Experimental mice were generated using paternal deficiency female mice as breeders, obtained from Jackson Laboratory (Bar Harbor, ME <https://www.jax.org/strain/016590>). Ube3a-deficient mice and WT littermates were obtained by crossing a paternal-null heterozygous female ($Ube3a^{m+/p-}$ mice) with a WT male, resulting in either WT progeny or progeny with loss of Ube3a only on the maternally inherited allele ($Ube3a^{m-/p+}$ mice).

All experiments were carried out in accordance with the European Directive of 22 September 2010 (EU/63/2010) and were authorized by the Italian Ministry of Health.

3.2 Chronic IOS imaging

3.2.1 Surgery

Surgery for IOS imaging was performed as described in Mazziotti et al. Mice were deeply anesthetized with isoflurane (1-3%), placed and head fixed on a stereotaxic frame using ear bars. Body temperature was controlled using a heating pad and a rectal probe to maintain the animals' body at 37°C. Local anesthesia was provided using subcutaneous lidocaine (2%) injection and eyes were protected with dexamethasone-based ointment (Tobradex, Alcon Novartis). The scalp was removed, the skull cleaned with saline and the skin was secured to the skull using cyanoacrylate.

Then, a thin layer of cyanoacrylate was poured over the exposed skull to attach a custom made metal ring (9mm internal diameter) centered over the binocular visual cortex. After the glue dried off, a drop of transparent nail polish was spread over the area to increase optical access. After surgery animals were placed in a heated box and monitored to ensure the absence of any sign of discomfort. Before any other experimental procedure, mice were left to recover for 24/48h. During this period, paracetamol (100mg/kg) was administered in the water as analgic therapy (Mazziotti et al. 2017).

3.2.2 Visual stimulation, acquisition and IOS setup

IOS recordings were performed under chlorprothixene (Dose) and isoflurane (1%) anesthesia. Images were visualized using an Olympus microscope (BX50WI). Red light illumination was furnished by 8 red LEDs (625 nm, Knight Lites KSB1385-1P) attached to the objective (Zeiss Plan-NEOFLUAR 5x, NA: 0.16) using a custom made metal LED holder (Harrison et al. 2009).

Animal was secured under the objective using a ring-shaped neodymium magnet (<https://www.supermagnete.it/>) mounted on an arduino-based 3D printed imaging chamber that controls eye shutters and a thermostated heating pad (<http://labrigger.com/blog/2015/11/30/open-source-intrinsic>) (Mazziotti et al. 2017). A schematic representation of the imaging chamber can be found in fig. 3.1[A].

Matlab Psychtoolbox were used to generate visual stimuli and presented on a monitor, placed 10 cm away from the eyes of the mouse. Sinusoidal wave gratings were presented in the binocular portion of the visual field (-10° to $+10^\circ$ relative to the horizontal midline and -5° to $+50^\circ$ relative to the vertical midline) with spatial frequency of 0.03 cyc/deg, mean luminance 20 cd/m² and

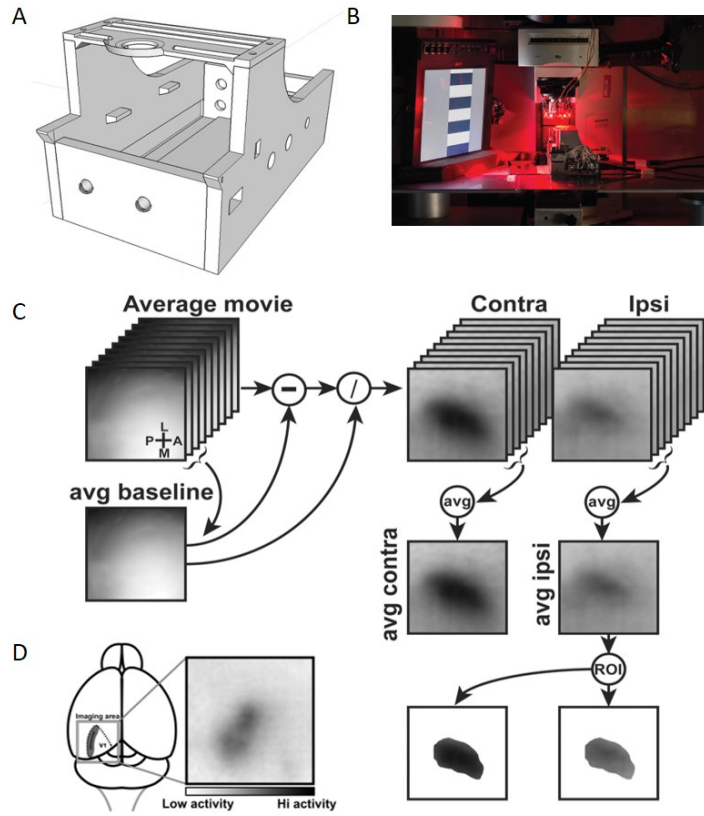


Figure 3.1: [A] Picture of the 3D printed imaging chamber [B] Picture of the setup [C] Flow chart of image processing for automated binocular visual cortex ROI generation. Frame size: 2×2 mm [D] Example of $\Delta R/R$ visual responses

contrast of 90% (Mazziotti et al. 2017).

The stimulus consisted in the abrupt contrast reversal of a sinusoidal grating with a temporal frequency of 4 Hz for 1 s, time locked with a 16-bit depth acquisition camera (Hamamatsu digital camera C11440) using a parallel port trigger (fig.3.1[B]).

Frames were acquired at 30 fps with a resolution of 512×512 pixels. A total of 210 frames were captured for each trial: 30 before the stimulus as a baseline condition and 180 as post-stimulus. The signal was averaged for at least 30 trials and downsampled to 10 fps. Fluctuations of reflectance (R) for each pixel were computed as the normalized difference from the average baseline ($\Delta R/R$) (Mazziotti et al. 2017). For each recording, an image representing the mean evoked response was computed by averaging frames between 0.5 and 2.5 seconds after stimulation. The mean image was then low-pass filtered with a 2D average spatial filter (30 pixels, $117 \mu\text{m}^2$ square kernel). To select the binocular portion of the primary visual cortex, a region of interest (ROI) was automatically calculated on the mean image of the response to ipsilateral stimulation by selecting the pixels in the lowest 30% ($\Delta R/R$) of the range between the maximal and minimal intensity pixel (Cang et al. 2005). The same ROI was used for the analysis of contralateral eye responses. A scheme of the data processing is shown in fig. 3.1[C]. Mean evoked responses were quantitatively estimated as the average intensity inside the ROI. ODI was calculated as $(C-I)/(C+I)$ for each pixel, where C and I indicate the amplitude of contralateral and ipsilateral responses, respectively (Cang et al. 2005). To weaken background fluctuations a manually selected polygonal region of reference (ROR) was subtracted. The ROR was placed where no stimulus response, blood vessel artifact or irregularities of the skull were observed (Heimel et al. 2007). Representative images of a typical

intrinsic signal response to visual stimulation localized in the V1 is shown in fig. 3.1[D] (Mazziotti et al. 2017).

3.3 Visually evoked potentials

3.3.1 Surgery

VEP recordings was performed as describe in Mazziotti et al. Anesthesia was induced in mice with 3% isoflurane; animals were then anesthetized with an intraperitoneal injection of urethane (150 mg/Kg; 20% solution in lactate ringer solution) and head-fixed in a stereotaxic frame. After the removal of the ring implant, the skull was exposed and a small craniotomy (2×2 mm) overlying the visual cortex was performed, leaving the dura mater intact. Then, a multichannel neural probe (Q-trode tetrode configuration, Neuronexus) was inserted at a depth of 150-200 μ m, in the binocular visual cortex (lambda:+3.2mm lateral,+0.2 mm rostral and following IOS derived coordinates), where a high amplitude positive VEP is detectable.

Signal was acquired using Cheetah 32 (Neuralynx) recording system at 30.3 kHz of sampling rate with a band pass filter of 0.1–250 Hz. Signal from each channel was averaged to obtain VEPs (Mazziotti et al. 2017).

3.3.2 Visual stimulation and data acquisition

Stimuli for VEP recordings were performed using custom software written in Matlab Psychtoolbox and displayed onto a gamma corrected 21 inch CRT monitor placed 20 cm in front of the animal (Mazziotti et al. 2017).

Stimuli consisted in contrast reversal square wave gratings with a spatial

frequency of 0.03 cyc/deg, mean luminance of 25 cd/m² and a contrast of 90%. Monitor was centered on animal head and stimuli covered 55° relative to vertical and -5°+30° relative to vertical midline (Mazziotti et al. 2017).

Stimulus temporal frequency was 2 Hz. VEP responses were averaged for at least 100 trials per eye. Each condition was repeated at least eight times. The amplitude quantification of the response was calculated as the peak to baseline difference of the first positive component (at ~ 100 ms). Visual acuity was calculated presenting contrast reversal sinewave gratings with different spatial frequencies (0.03, 0.06, 0.012, 0.024, 0.048, 0.96 and 0 cyc/deg) and calculating the maximal spatial frequency resolved by linear fit estimation of parameters and extrapolation of amplitude at $0\mu\text{V}$ in a semi logarithmic space. Contrast sensitivity was calculated using 0.03 cyc/deg contrast reversal sine wave gratings with different contrasts (90, 50, 25, 12, 6, 3 and 0%). Normalized amplitude data was fitted to a sigmoid curve using Naka–Rushton function, $\text{Resp}(c) = 1C^n / (C50^n + C^n)$, to extract contrast sensitivity parameters (D. G. Albrecht and Hamilton 1982; Sarnaik et al. 2014). C50 is the contrast evoking half the maximum response and n describes the shape of the curve (Mazziotti et al. 2017).

3.4 Appetitive conditioning chamber

3.4.1 3D-printed OC chamber

The OC arena ($16 \times 16 \times 16$ cm; thickness, 3 mm; fig.3.2 [A]) is 3D printed using gray or white PLA (B06W568X1G, Technology Outlet). The 3D project is designed using FreeCAD software, exported in stereolithography (STL) for-

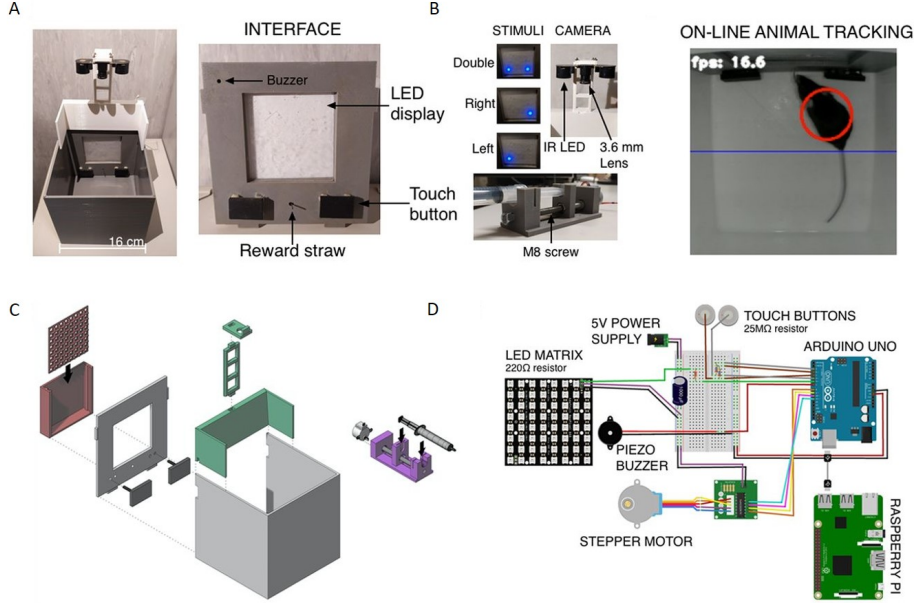


Figure 3.2: [A] left, Top-view of the apparatus. Center, Interface wall. Right, Blue dotted stimuli, camera holder, and syringe pump. [B] An animal during the task, the blue line delimitates the “active zone.” [C] An exploded view of the project showing the assembling scheme. [D] Circuit diagram of all the components.

mat, converted to G-code using Cura (<https://ultimaker.com/software/ultimaker-cura>) and printed using Kentstrapper Verve 3D printer (<https://kentstrapper.com/stampante-3d-kentstrapper-verve/>) (Mazziotti et al. 2020a).

In fig.3.2 [C], an exploded-view drawing of the chamber is shown. The color coding corresponds to different components of the apparatus (visual stimulation parts in red; camera holder in green; syringe pump in purple). All these components are coated using epoxy transparent resin (LF-L2GR-26GX, resinpro), which allows cleaning (5% ethanol in water). The arena front wall contains

the elements interfacing the animal with the computer. It can be modularly assembled to the arena and is composed by a squared frame containing the LED matrix at the center, four holes for the touch buttons in the lower part, a central hole for the lick spout and a hole in the upper part to connect a piezo buzzer. The touch buttons are printed using graphene PLA (PLA_GRAFENE_175, filoprint), and connected using conductive glue (Chemtronics: CW2400) to a female pin (B07XQHD752, amazon.it) using a resistor (25 M Ω) (Mazziotti et al. 2020a). A dotted grid is interposed between the LED matrix and the inside of the chamber and has two roles: first, the dotted pattern restricts the visibility of the LED lights to equal small circles; second, it contains a grid of walls facing the LEDs that prevents the light from each source to spill over to the neighboring dots. The LED matrix is covered with a thin white Plexiglas foil, so that single LED are not visible if they are off and to diffuse light uniformly. The camera holder is jointly assembled on top of the frontal wall and it is designed to maintain the camera at the distance necessary to image the entire arena using a 3.6 mm focal length objective. The syringe pump is composed of a base that fixes the barrel of the syringe into position and of a piston that slides on a stepper motor guided M8 metal screw and allows it to push or pull the plunger (Mazziotti et al. 2020a).

3.4.2 Hardware

An electronic board is mounted on a grounded metal sheet and is composed by a Raspberry Pi connected via USB to an Arduino UNO (AU) board (<https://store.arduino.cc/arduino-uno-rev3>). The Raspberry Pi (<https://www.raspberrypi.org/products/raspberry-pi-3-model-b/>) acts as the main computer of the setup. It executes the Python three script that handles the structure

of the experiment, performs computer vision using a Raspberry PI camera (Bewinner: Bewinnertyv48w6mf5), and saves data (Fig. 3.2[B]).

The AU controls sensors and actuators in the OC chamber. Two touch buttons, made using conductive PLA, acts as capacitive sensors and are connected to AU using coaxial cables (3 mm in diameter) to minimize environmental noise. The main advantage of using graphene PLA resides in the possibility to print different button designs (e.g., for motor impairment, nose poking, etc.). There are three actuators: a LED matrix serves as display (Adafruit: 1487), a piezo buzzer (Adafruit: PS1240, frequency range: 2–10 kHz, 60 dB) is used as acoustic stimulator glued at the top of the frontal door, and a stepper motor (amazon.it: 28BYJ-48, with ULN2003) connected to a M8 screw guiding the piston of a syringe pump controlling a disposable syringe (10 ml) connected with a silicone tube equipped by luer tapers adapters to a blunt needle (Warner Instruments: SN-18) for reward delivery. This modular configuration allows the proper cleaning of the delivery tubing after each session. I use an external 5-V 2-A DC power supply (Samsung: TA10EWE) with a 1000 - μ F capacitor to power the LED matrix and the stepper motor. A diagram of the electrical wiring is shown in the fig 3.2[D].

3.4.3 Software

AU program

The code controlling the OC box is organized in four files, the file called `skinner.ino` contains the logic of the experiment and manages the serial communication with the computer (Mazziotti et al. 2020a).

Different files are dedicated to different aspects of the program: the file

called `button.ino` contains functions to control the touch buttons and play auditory stimuli, the file called `ledLib.ino` contains wrapper functions to control Adafruit NeoPixel library (<https://www.adafruit.com/product/1487>) and generate simple visual stimuli easily, the third file called `stepper.ino`, contains functions to control the syringe pump using the Arduino Stepper motor library (<https://www.arduino.cc/en/reference/stepper>). In summary, to set up the AU, a user needs to download the folder containing the `.ino` files, uncompress and upload the file `skinner.ino` (Mazziotti et al. 2020a).

Raspberry Pi program

On the Raspberry Pi, a Python script controlling the experiment has been written using IDLE. The program relies on a number of external libraries that are required to run all parts of the script with no errors. Since the task relies on real time tracking of the animal position I use `picamera` and `opencv` libraries to acquire frames and process them using K-nearest neighbors based background-foreground segmentation (Zivkovic and Van Der Heijden 2006), a widely used algorithm for generating a foreground mask using static cameras fig 3.3 [A](Mazziotti et al. 2020a).

The technique consists of two main steps, the first one is the background initialization in which I use 1000 frames of the empty arena, then I set the learning rate to zero and the algorithm stops updating the background so it is ready to locate reliably the position of the animal with a frame rate of 20 Hz. `LibSerial` library is used to communicate with the AU during the task, sending symbolic codes and changing the state of the AU in the OC chamber. I used `Tkinter` library to write the initial GUI to set the experimental parameters (Mazziotti et al. 2020a).

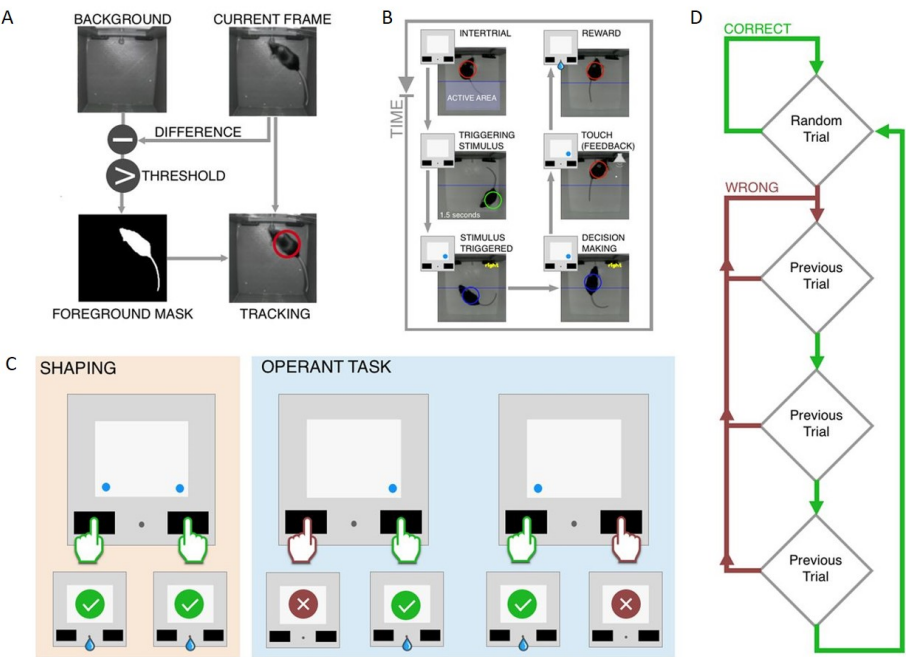


Figure 3.3: [A] The detection of the mouse is obtained using background subtraction from the current frame and then applying a threshold, isolating only the mouse silhouette [B] Behavioral sequence to obtain the reward. [C] Diagram showing the behavioral procedures, during the shaping phase and the operating task. [D] Flowchart of the assisted procedure.

The behavioral sequence is outlined in fig 3.3 [B] Virtually the chamber can be divided into two sections: the anterior part that contains the interface between the mouse and the computer, and the posterior side that is designed as an active area to activate the trials. If the mouse remains in the active area for a given amount of time (1.5 s) the trial is triggered. At this stage a visual stimulus is shown on the display and the system waits for animal response. When the mouse touches one of the two buttons, an auditory feedback is produced, with a tone that varies depending on whether the answer is correct (3300 Hz) or wrong (2700 Hz). In case of correct answer, a drop (7 μ l) of water with 1% condensed milk is released (Mazziotti et al. 2020a).

3.4.4 The OC protocol

Familiarization (1 d)

This phase is conducted by placing each animal in the OC box for three sessions of 10 min, spaced by at least 2 h between each other. During this phase, a liquid reward, coupled with the “correct” tone is provided manually whenever the mouse is in the active area, in this way the animal learns where to find the reward and associate it with the tone (Mazziotti et al. 2020a).

Shaping (3 –4 d)

The visual stimulus is introduced Fig. 3.3 [C] It consists of two bright (0.9 cd/m²) blue (465 –475 nm) dots (5 mm) that appear above the two buttons after stopping for 1.5 sec in the activation area. The mouse needs to touch one of the two buttons to obtain the reward (Mazziotti et al. 2020a).

Operant task (OT, 5 d)

During this phase, only one dot appears, identifying the correct button. If the mouse touches the correct button, the correct tone is reproduced and the animal receives the reward. If the mouse touches the wrong button, the “wrong” tone is reproduced and no liquid reward will be delivered. This procedure is shown in fig.3.3 [C] The sequence of stimuli is balanced so that the mouse sees each case the same number of times. In order to prevent perseveration with the same answer, during the first 2 d I adopted an assisted procedure (fig. 3.3 [D]): the first stimulus presented is random, if the mouse produces the correct answer the following stimulus is randomized, in case of wrong answer instead, the system repeats the same stimulus until the mouse gives the correct answer three times.

Delayed (3 d)

To evaluate cognitive flexibility, I slightly modified the shaping phase, leaving the stimulus unchanged, but doubling the time spent in the activation area (from 1.5 sec to 3 sec).

3.5 Viral injections and therapy administration**3.5.1 DREADD injection in PFC**

Mice were deeply box-anesthetized and maintained with isoflurane (respectively, 3 and 1%), placed on a stereotaxic frame and head fixed using ear bars. Body temperature was controlled using a heating pad and a rectal probe to maintain the animals' body at 37°C. Local anesthesia was provided using subcutaneous lidocaine (2%) injection and eyes were protected with dexamethasone-based

ointment (Tobradex, Alcon Novartis).

Mice received injections of pAAV -hSyn-hM4D(Gi)-mCherry (7×10^{12} vg/mL, Addgene). A micromanipulator (GUARDA MARCA) and calibrated micro-capillaries were used to inject 0.3 μ L of virus (pAAV -hSyn -hM4D(Gi)-mCherry and (altro virus di controllo) in control group) bilaterally in medial prefrontal cortex (mPFC) (coordinates from bregma: anterior/posterior +2.0 mm, medial/lateral $\pm$ 0.3 mm, dorsal/ventral -1.8 mm). After surgery the animals were placed in a heated box and monitored to ensure the absence of any sign of discomfort. Before any other experimental procedure, mice were left to recover for 24/48 h. During this period, paracetamol (5 mg/ml) was administered in the water as analgic therapy.

3.5.2 Cyclocreatine treatment

Cyclocreatine (cCr) was dissolved in 1% low-fat chocolate milk. Dosing solutions were stored at 2–8 °C between use and fresh solutions were made weekly. CCr in 1% chocolate milk was shown to be stable for 3 weeks at room temperature, and solubility was determined to be 15 mg/mL.

Animals were left untreated or presented daily with 1% low-fat chocolate milk (vehicle treatment, V) or a 1% low-fat chocolate milk-cCr cocktail (in a volume of 5 ml/kg). cCr was administered once daily at three different dose levels (140, 46 or 14 mg/kg) over the course of 24 weeks starting in mice at postnatal day (PND) (Cacciante et al. 2020a).

3.5.3 Statistical analysis

For the assessments in mice, we estimated the sample size needed for each experiment by performing a power analysis using G*Power ($\alpha = 0.05$, $\beta = 0.2$). we estimated the minimal difference that would be biologically relevant considering the impact of the possible difference on the animals' brain function and behaviour. All statistical analyses of the results were performed using GraphPad Prism 8.0.1 and Python. All values were presented as mean $\pm$ SEM unless otherwise stated. For analysis of two groups were used t-test for a difference in mean involve independent samples (unpaired samples). The significance of factorial effects and differences among more than two groups were evaluated with ANOVA/repeated measures ANOVA followed by post hoc Holm–Sidak test. The level of significance was $P < 0.05$.

Chapter 4

Results

4.1 The visual system as a biomarker of NDDs

In this section, I will present the results of visual evoked amplitude responses, in different NDDs, such as CDKL5, creatine transporter deficiency, Krabbe disease, and Angelman syndrome. As mentioned above, I used IOS imaging, a functional non-invasive technique, based on BOLD signal, and VEP, to monitor the visual responses in these pathologies. There is a pressing need for biomarkers of high translational value to noninvasively and quantitatively monitor brain function during the progression of the disorder and treatment. The visual system and, in particular, the amplitude of visual cortical responses represents a promising biomarker for preclinical and clinical studies. Moreover, recent literature is evaluating the connection between energy metabolism and neurodevelopmental disorders. Energy metabolism is essential for normal brain function and development, and defects in brain metabolism result in neurodevelopmental diseases

(Oyarzábal et al. 2021). The review evaluates a series of monogenic disorders, such as Creatine transporter deficiency, Rett, X-fragile and Down syndrome, autism, schizophrenia, and epileptic encephalopathies, that show a common underlying metabolic disorder (Oyarzábal et al. 2021). The strict energetic demands of the brain require that nutrient supply and usage be fine-tuned by the specific temporal and spatial patterns of ever-changing levels of neuronal activity. This is achieved by adjusting local cerebral blood flow (CBF) as a function of activity level - neurovascular coupling - and by changing how energy substrates are metabolized and shuttled amongst astrocytes and neurons - neuroenergetic coupling (Lourenço et al. 2017). IOS imaging allows measuring the neurovascular coupling, and, indirectly, the energy metabolism in these pathologies. For these reasons, I evaluate cortical visual amplitude in these pathologies, hoping to find a common output of energy metabolism disorder and a reliable biomarker.

4.1.1 CDKL5 deficiency disorder

To evaluate the impact of CDKL5 mutation on visual cortical development I examined cortical responses to visual stimulation using intact skull repeated IOS imaging in young mice and tracking response maturation in binocular visual cortex during visual development (P25–32) (Espinosa and Stryker 2012).

I examined IOS responses in the same subjects, both male CDKL5 null and female HET mice compared with their age and sex-matched WT littermates, at three different developmental stages as reported in Fig 4.1

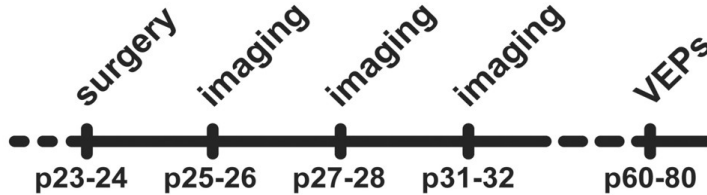


Figure 4.1: Sketch of the experimental protocol

IOS imaging during development reveals decreased amplitude of cortical visual responses in CDKL5 mice

Data quantification showed that at the first age tested (P25–26) in male mice, the amplitude of the responses to contralateral eye stimulation was comparable between CDKL5 null and WTm, however, starting from the second time point (P27–28 and P31–32) KO mice showed a significantly reduced response concerning WTm (fig. 4.2 [A]). The responses to the ipsilateral eye showed a similar pattern: no difference between genotypes at P25–26 and significantly reduced amplitude in CDKL5 null mice at P27–28 and P31–32 (fig. 4.2 [A]). No change in ODI was present in null mice with respect to controls (fig. 4.2 [B]) (Mazziotti et al. 2017).

Interestingly, female heterozygous CDKL5 mice also showed a similar developmental impairment of contralateral evoked responses, but preserved ipsilateral response amplitude (fig. 4.3 [A]). This differential effect on ipsilateral and contralateral responses resulted in an abnormal ocular dominance index (ODI) biased toward ipsilateral eye in HET mice with respect to WTf (fig. 4.3 [B]) (Mazziotti et al. 2017).

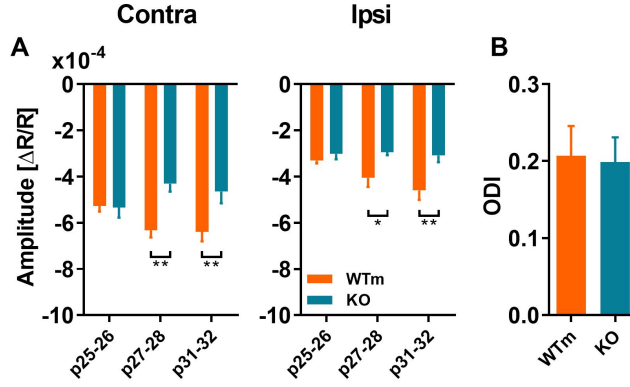


Figure 4.2: [A] Respectively controlateral (contra) wt N = 9, CDKL5 null N = 7; two-way ANOVA genotype P = 0.035, post hoc Holm-Sidak wt vs mutants P25-26 P = 0.91, P27-28 P = 0.0016, P31-32 P = 0.0047 and ipsilateral (ipsi) wt N = 9, CDKL5 null N = 7; two-way ANOVA genotype P = 0.0017, post hoc Holm-Sidak wt vs mutants P25-26 P = 0.5, P27-28 P = 0.024, P31-32 P = 0.0028 amplitude response during development in WTm and KO mice, [B] ODI in WTm and KO, wt N = 9, CDKL5 null N = 7; t-test P = 0.87

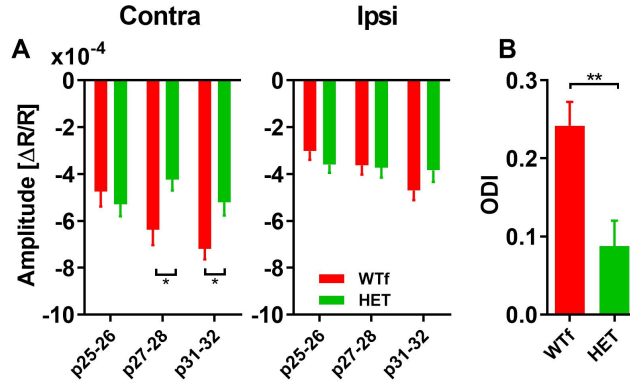


Figure 4.3: [A] Respectively controlateral (contra) wt N = 11, CDKL5 HET N = 7; two-way ANOVA time $\times$ genotype P = 0.017, post hoc Holm-Sidak wt vs mutants P25-26 P = 0.53, P27-28 P = 0.046, P31-32 P = 0.049; and ipsilateral (ipsi) two-way ANOVA time $\times$ genotype P = 0.17, genotype P = 0.87 amplitude response during development in WTf and HET mice; [B] ODI in WTf and HET; WTf N = 11, CDKL5 HET N = 7; t-test P = 0.003

VEP recordings confirm the visual impairment

To investigate whether the visual impairment detected during developmental persists into adulthood I recorded VEPs in P60–P80 mice. To establish whether the defect observed in juvenile mice was correlated with adult visual cortical responses, I evaluate the same mice that were studied during development through IOS imaging, a non-terminal procedure that allows recovery of the animal at the end of the procedure. I found a reduction of VEP amplitude in adult KO male mice for both contralateral and ipsilateral eye stimulation (fig. 4.4 [A]) (Mazziotti et al. 2017).

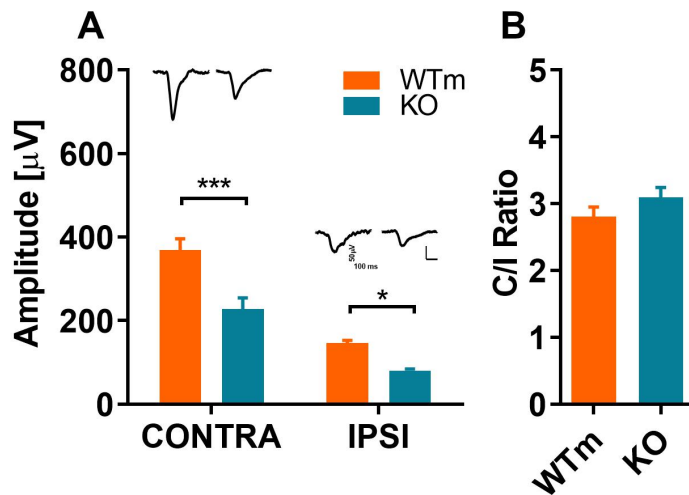


Figure 4.4: [A] VEP amplitude of adult male mice, wt N = 6, KO N = 6; two way ANOVA genotype $P < 0.0001$, post hoc Holm–Sidak wt vs mutants Ipsilateral eye $P = 0.028$, Contralateral eye $P = 0.0001$; [B] C/I ratio in WTm and KO, wt N = 6, CDKL5 null N = 6; t-test $P = 0.22$

Similarly, contralateral, but not ipsilateral, eye responses were significantly

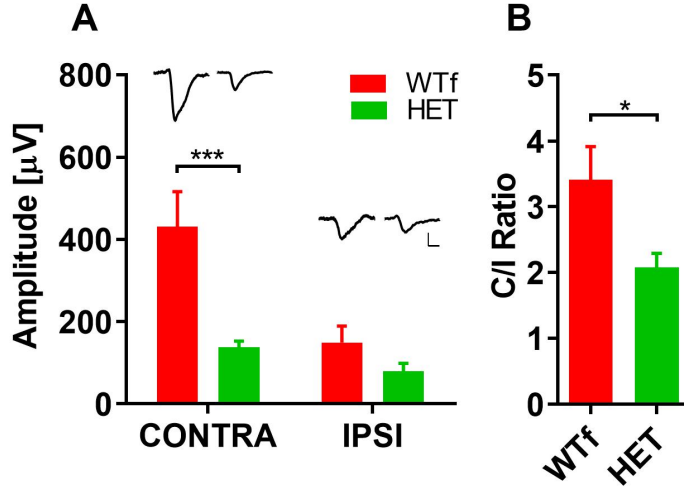


Figure 4.5: [A] VEP amplitude of adult female mice, wt N = 8, HET N = 8; two way ANOVA eye $\times$ genotype P = 0.029, post hoc Holm-Sidak wt vs mutants Ipsilateral eye P = 0.31, Contralateral eye P = 0.0004 [B] C/I ratio in WTf and HET, wt N = 8, CDKL5 het N = 8; t-test P = 0.03

reduced in HET females (fig. 4.5, [B]) resulting in an abnormal ODI in CDKL5 heterozygous mice (fig. 4.5 [B]), but not in CDKL5 null males (fig. 4.4 [B]). These data, obtained in adult mice, overlap with the results found during early development using IOS imaging (Mazziotti et al. 2017).

CDKL5 loss in excitatory cells is sufficient to produce the visual deficit

CDKL5 mutant mice present a deficit in the processing of visual stimuli that can be assessed with a remarkably high discrimination power by IOS imaging of visually evoked responses (Mazziotti et al. 2017). These abnormal visual responses, however, could arise from defects in upstream visual areas with no intrinsic dysfunction present in visual cortical circuits.

To assess the specific contribution of the visual cortex, I used a conditional KO mouse model in which the removal of the floxed CDKL5 allele in mutant mice (CDKL5^{fllox/y}) was achieved by crossing these mice with mice expressing CRE-recombinase driven by the *emx1* promoter (*emx1*-CRE^{+/-}). As a result, CDKL5^{fllox/y}; *emx1*-CRE^{+/-} animals (cKO) lack CDKL5 only in cortical excitatory neurons and in some glial cells (Gorski et al. 2002), thus sparing all the subcortical visual areas. The expression of CRE-recombinase driven with the *emx1* promoter is known to induce recombination as early as at embryonic day (E) 10.5 (Gorski et al. 2002), a time point in which the expression of all the most abundant isoforms of CDKL5 in the mouse brain is still very low (Hector et al. 2016). This ensures that the phenotypic effects observable in adult mice are the result of an altered development or maturation, closely resembling CDKL5 null mice and patients.

To explore the role of cortical CDKL5 in the pathogenesis of visual deficits in CDKL5 mutants, I first asked whether the morphological alterations previously identified in germline CDKL5 null mice were present in cKO mice in which CDKL5 is normally present in subcortical visual nuclei. Since the retina is unaffected in CDKL5 germline mutants, I began evaluating the presence of such alterations in the visual pathway of cKO mice from the dLGN. Spine density and morphology, PSD95+ puncta and VGAT+ puncta were analyzed in cKO and WT littermates. The results showed that spine density in the dLGN of cKO mice was not different from WT control mice (Lupori et al. 2019). Moreover, both the proportion of mature and immature spines and their morphology were unaltered (Lupori et al. 2019).

Finally, the density of PSD95+ and VGAT+ puncta did not differ between WT and cKO mice (Lupori et al. 2019). Thus, as expected from the pattern

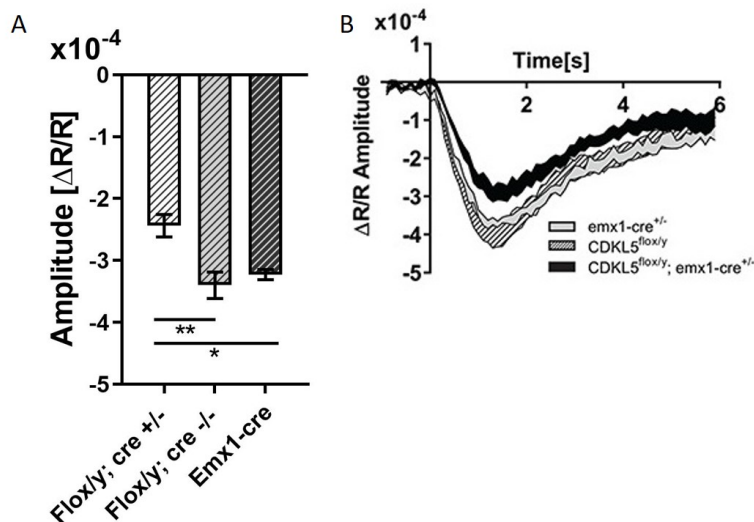


Figure 4.6: [A] Quantitation of the average amplitude of IOS between cKO, floxed controls (CDKL5^{flox/y}) and emx1-CRE driver controls (emx1-CRE^{+/-}), one-way ANOVA $p = 0.004$; post hoc Holm-Sidak; emx1-CRE^{+/-} vs cKO; $P = 0.0342$, CDKL5^{flox/y} vs cKO; $P = 0.004$. [B] Average time course of the intrinsic signal in the V1 after visual stimulation

of expression of Cre recombinase, the cKO model preserves the integrity of the subcortical retino-cortical pathway, allowing to study the role of cortical CDKL5 in the genesis of visual alterations.

I then asked whether cortical CDKL5 plays a necessary role in the normal physiology of cortical visual processing. To this purpose, I performed IOS imaging, recording visually evoked responses in male cKO animals ($N = 8$) and, as a control, in parental mouse lines expressing CRE ($N = 5$; emx1-CRE^{+/-}) or carrying a CDKL5 floxed allele ($N = 9$; CDKL5^{flox/y}; Fig. 4.6). IOS response amplitude to visual stimulation was significantly different between groups (Fig. 4.6 [A]). cKO animals showed a decreased response amplitude with respect to

both CRE-expressing mice and CDKL5 floxed mice. Such a reduced response is also evident in the average time course of the cortical reflectance for each group (Fig. 4.6 [B]). Interestingly, by quantitatively measuring the magnitude of the impairment using Cohen's d as an effect size estimator (Cohen 2013), I noticed that the effect produced by the germline deletion of CDKL5 was bigger (Cohen's $d = 2.78$) than the one produced by conditional deletion from excitatory cortical cells (Cohen's $d = 1.73$), although the effect size can be regarded as very large for both CDKL5 null mice and cKO (Sawilowsky 2009). Thus, the effect size analysis suggests a major role for the cortex in generating the visual phenotype although it cannot be excluded an enhancement of the deficit by defects in other brain areas or cell types.

4.1.2 Creatine transporter deficiency disorder

I evaluated cortical amplitude responses in order to identify novel, translational and non-invasive biomarkers for the analysis of brain function in creatine transporter deficiency, providing a very reliable protocol to longitudinally monitor the efficacy of potential therapeutic strategies in preclinical, and possibly clinical, studies (Mazziotti et al. 2020b).

Altered neurovascular coupling in KO mice

I examined cortical responses to visual stimulation using IOS imaging in juvenile (P 40) CrT-/y animals. Data analysis revealed that IOS amplitude of the responses driven by contralateral eye stimulation was significantly higher in CrT-/y compared to control mice and the effect was particularly evident at higher contrasts of visual stimuli. I longitudinally tracked visual responses in the same animals as they aged using the 90% contrast visual stimuli. The response to the contralateral eye showed a similar pattern at P 110 and P 180 with a significantly increased amplitude in CrT-/y compared to WT mice (Fig. 4.7 [A]). These results show that hemodynamic response is markedly altered in the cortex of mutant animals and that this phenotype progresses with age. To clarify whether this is related to a corresponding change of ongoing neuronal activity, I assessed visual responses in a separate group of adult (P 110) CrT-/y mice using intracortical VEP recordings. Data quantification revealed comparable visually evoked potential amplitude and latency in CrT-/y and WT animals (Fig. 4.7 [B]).

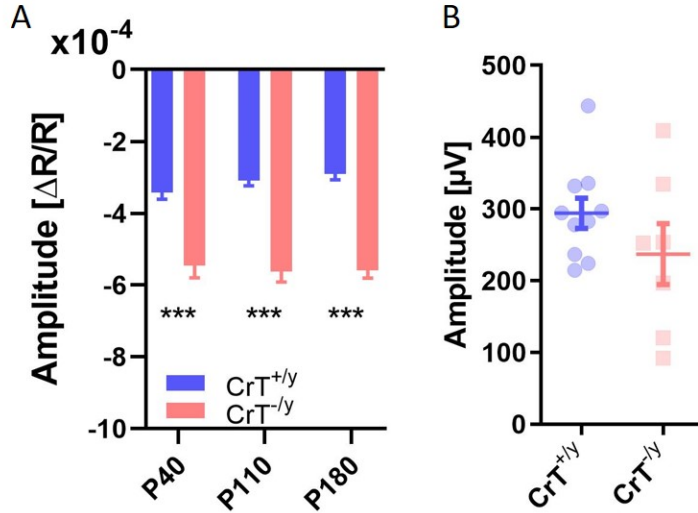


Figure 4.7: [A] Response for contralateral eye stimulation in male WT and CrT^{-/y} mice at three different time points, P 40, P 110 and P 180; $n = 15$ for each group at P 40 and P 110, $n = 19$ at P 180. two-way ANOVA, effect of genotype $P < 0.001$, post hoc Holm-Sidak, $P < 0.001$ at all ages tested. [B] Visually evoked potential amplitude, WT $n = 10$ and CrT^{-/y} animals $n = 7$; t-test, $P = 0.767$

Creatine conditional KO produce the visual alteration detected in total KO

In order to dissect the role of neural Cr in CTD and in particular in the visual deficit, I evaluated the visual amplitude responses in two different conditional KO of creatine mouse model, in particular, I used a nervous system-specific model, the Nestin::Cre recombinase Tg (Nes-cre) 1Kln mouse, and subsequently the Parva::Cre recombinase (Parva-cre) mouse. Molinaro et al. report that nervous system-specific Cr depletion (Nes-cre) leads to a progressive cognitive regression starting in the adult age. No autistic-like features, including repetitive and stereotyped movements, routines, and rituals, are present in this model. These

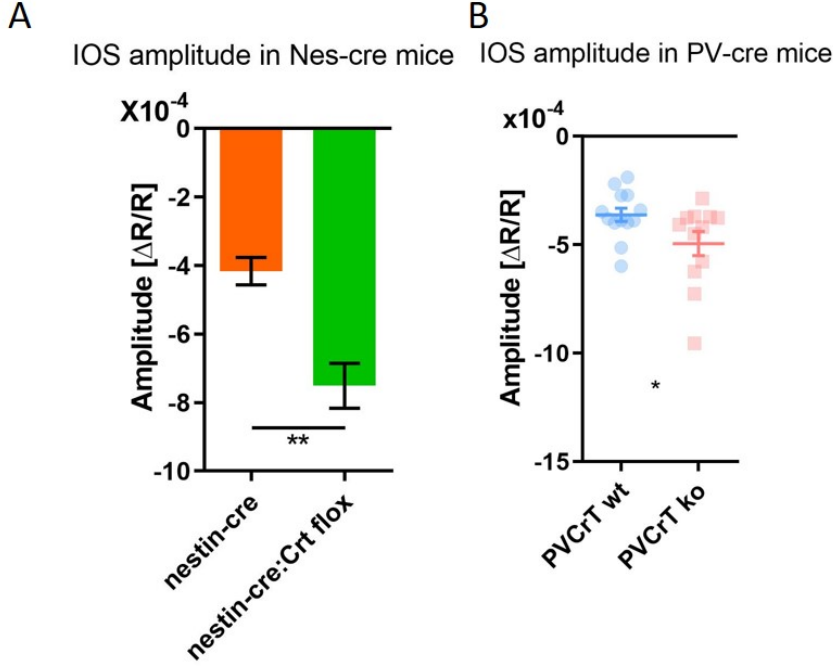


Figure 4.8: [A] Nes-cre mice show increased amplitude (Nestin-cre wt N = 6, nestin-cre:Crt flox KO N = 6; t-test = 0.0022. [B] PVCrT mice exhibit an increase amplitude in visual responses (PVCrT wt N = 13, PVCrT KO N = 12; t-test = 0.0443)

results indicate that Cr depletion in the nervous system is a pivotal cause of the CTD pathological phenotype, in particular with regard to the cognitive domain, but extraneural actors also play a role. I subsequently evaluate IOS amplitude responses in Nes-cre mice, finding exactly the same deficit detected in total KO as reported in Fig. 4.8 [A]. Moreover, I evaluate a more specific conditional ko, the PVCrT mice, in which the lack of CrT is restricted to parvalbuminergic neurons, finding a mild, but comparable phenotype in PVCrT KO mice 4.8 [B].

cCr administration improves cortical hemodynamics

Since I reported that hemodynamic responses are altered in the visual cortex CrT-/y mice with no impairment of visually-evoked potentials and that hemodynamic measurement are a reliable biomarker of brain function in CTD (Cacciante et al. 2020a), I explored the therapeutic efficacy of cyclocreatine (cCr) using IOS imaging. cCr was administered orally once daily at three different doses (high dose, H: 140 mg/kg, medium dose, M: 46 mg/kg, low dose, L: 14 mg/kg) to CrT-/y mice and wild-type littermates starting at P 21. I longitudinally evaluated the therapeutic efficacy of cCr treatment in the same animals. I found a dose- and age-dependent effect on hemodynamic responses in CrT-/y mice administered cCr. The altered IOS amplitude observed in CrT-/y mice was significantly attenuated in CrT-/y mice treated with the high dose of cCr (H-CrT-/y) at every time point tested. The medium dose (M-CrT-/y) ameliorated IOS signals at P 110 and P 180, and the low dose (L-CrT-/y) was effective only at P 180 4.9 [A, B]. No difference was detected between untreated animals (CrT-/y) and vehicle-treated mice (V-CrT-/y, 4.9 [A, B]). These data suggest that cCr delivery is able to circumvent, at least partially, the metabolic impairment caused by Cr depletion, leading to the reversion of the neurovascular phenotype characterizing CrT-/y mice.

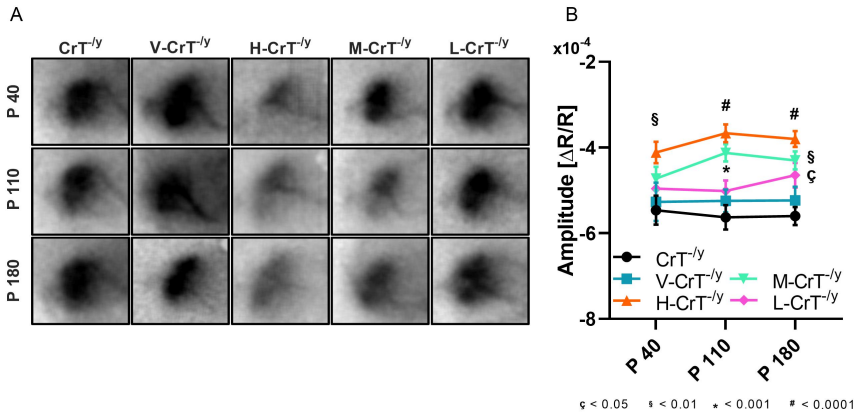


Figure 4.9: [A] Representative IOS response images collected at different ages (P 40, P 110 and P 180) in CrT^{-/-}, V-CrT^{-/-}, H-CrT^{-/-}, M-CrT^{-/-} and L-CrT^{-/-} mice ($n = 15$ for each group at each time point, except $n = 17$ each for CrT^{-/-}, H-CrT^{-/-} and M-CrT^{-/-} mice at PND180). [B] Mean amplitude of visually evoked IOS responses measured after 20 (P 40), 90 (P 110) and 160 (P 180) days of cCr delivery. Two-way ANOVA revealed a significant effect of treatment ($p < 0.001$) and post-hoc Dunnett showed an amelioration of hemodynamic responses in H-CrT^{-/-} mice at every time point tested ($p < 0.01$ at P 40, $p < 0.001$ at P 110 and P 180), whereas IOS signals were attenuated at P 110 ($p < 0.001$) and P 180 ($p < 0.01$) in M-CrT^{-/-} animals, and only at P 180 ($p < 0.05$) in L-CrT^{-/-} mice. No difference was present between untreated CrT^{-/-} animals and V-CrT^{-/-} mice ($p = 0.965$ at P 40, $p = 0.728$ at P 110, $p = 0.739$ at P 180). Error bars, SEM.

4.1.3 Other neurodevelopmental disorders

To establish the ability to detect visual alteration in other neurodevelopmental disorders, I evaluate cortical visual amplitude in Krabbe disease and Angelman syndrome. The possibility to identify biomarkers in different disorders allows us to precisely monitor the trajectory and possibly the outcome of therapeutics medication. Moreover, following the review of Oyarzábal et al., both of these monogenic neurodevelopmental pathologies could manifest energy demand disorders, that IOS imaging can detect.

IOS imaging detect alteration in twitcher and UBE3A mice

I evaluate visual amplitude response with IOS imaging in Twitcher homozygous mice compared to WT littermates. I evaluate mice at p 30, at this age, the pathological phenotype is already manifest but vision is still preserved.

As is report in fig. 4.10 [B], I detected a visual amplitude response alteration similar to cRt deficiency, with higher amplitude detected in Hom mice. Interestingly, I detected the same alteration, reported in fig 4.10 [A], in P 90 UBE3A heterozygous mice compared to WT littermate.

These results show that hemodynamic response is markedly altered in the cortex of mutant animals, both in Angelman syndrome and Krabbe disease, suggesting that this neurovascular alteration, could arise from a defect in energy metabolism.

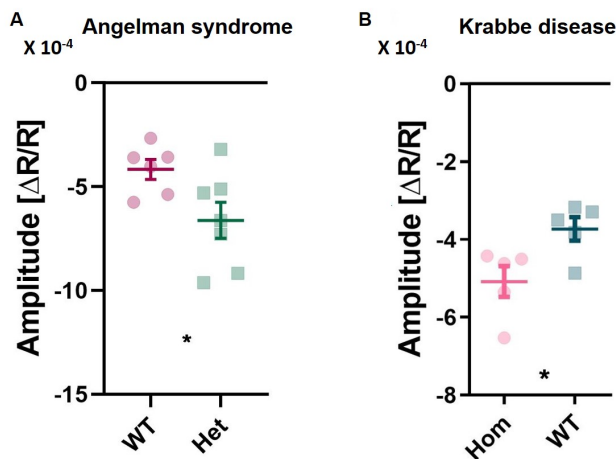


Figure 4.10: [A] Amplitude response to visual stimulation detect with IOS imaging (wt N = 6, Het N = 7; t-test = 0.0382; [B] Amplitude response to visual stimulation detect with IOS imaging (wt N = 5, Hom N = 5; t-test = 0.0265)

4.2 Learning curves in mice during OC test

In the previous section, I established the visual system as a biomarker of disease progression and for monitoring therapeutic approaches. Nevertheless, there is a lack of behavioral biomarkers capable, in the same way, of monitoring behavioral parameters quantitatively, an-bias, and longitudinally. For this reason, I developed and validated a novel tool based on operant conditioning (OC). OC is a classical paradigm and a standard technique used in experimental psychology in which animals learn to act to achieve a reward. I developed this behavioral tool to used this method as a reliable and unbiased test able to break down the variability given by the experimenter. As reported in the previous chapter, the OC chamber is a completely automated tool, using this paradigm, it is possible to extract learning curves and measure accurately reaction times (RTs). Measuring

these parameters allow to identify a behavioral biomarker, and provide unbiased measures of behavioral index that are proxy of cognitive capabilities and can be used to evaluate the effectiveness of therapeutic interventions in mouse models of disease (Mazziotti et al. 2020a)

4.2.1 Validation of the chamber in WT mice

Behavioral performance

To test the system's ability to detect learning curves, I trained six wild-type C57BL/6J (P50 to P180, four female and two male mice) as reported in Fig. 4.11 [B]. In the shaping phase, the average number of trials (TR) progressively increases overtime for all the subjects, and specifically on the third day, I detect a significant increase compared with the first day. Moreover, RTs and intertrial intervals (ITIs) showed a similar trend with a significant reduction of the RT starting from the second day (Fig. 4.11 [A]). This indicates that already at day 2, the animals started to refine the sequence of actions necessary to trigger the stimulus and produce a response. Next, the results of the OT phase are shown in Figure 4.11 [C]. The average TR continued to grow until day 3 of OT. After this day, the majority of the animals performed the maximum TR permitted in each session. Both RT and ITI showed a decrease with time. Indeed, RT and ITI dropped significantly during the first 2 d and reached a plateau by the third day. I observed a significant difference in the percentage of correct responses between the first and second days. In order to assess the retention of the test over time, I tested the same mice at different time points after the end of the OT. Accuracy remained stable during all time points tested; however, RT showed a more complex pattern: with an initial decrease, compared with the

last day of the OT, followed by an increase at four months. Analyzing ITI, I detected an increase of the time between two trials at 27 d that remained higher at three and four months compared with the end of the OT (Fig. 4.11 [D]). It is interesting to note that, since touch sensors are activated from all sides, some of the variability in timing performance could be explained by the development of different strategies to activate sensors (Mazziotti et al. 2020a).

Tracking analysis

In Figure 4.12 [A], tracking traces, of all the mice, are shown with corresponding heatmaps, averaged across animals (on the right) or days (bottom row), showing non-uniform exploration of the OC chamber during tasks. Pixels that were not significantly explored compared with randomly resampled uniform exploration values ($p > 0.05$) were set to 0. The reward area was the most visited place, as shown by both the animals and session average heatmaps. In the bottom half of the arena, there are two significant exploration spots at the corners, that indicate a stereotyped strategy to activate the trial (Fig. 4.12) [B]. Moreover, I analyzed the distance traveled by each animal inside the OC box during all the tasks. I found that, throughout the course of the shaping phase, there was a significant decrease in the total amount of distance traveled compared with the first day (Fig. 4.12 [B]). Conversely, during the OT phase, I detected no changes ($p = 0.3672$). Interestingly, I found a significant correlation between timing performances and the total distance moved during shaping (Fig. 4.12 [C]), this suggests that $\sim 25\%$ ($r^2 = 0.247$) of the improvement in timing performance is explained by a reduction in the distance traveled and the response of the animal. In addition, no differences in the average speed were detectable during both shaping and OT. These results imply that the reduction of the RT is due to the

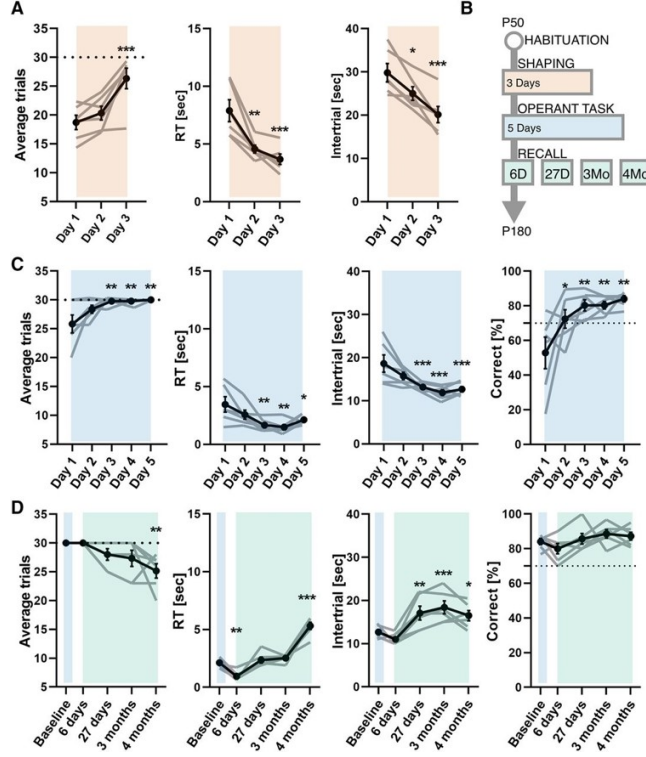


Figure 4.11: [A] Shaping phase: RM one-way ANOVA, Dunnett's multiple comparisons post hoc; average trials: $p = 0.0006$, post hoc day 1 vs day 3, $p < 0.001$; RT: $p = 0.0002$, day 1 vs day 2, $p = 0.001$ and day 1 vs day 3, $p = 0.0002$; ITI: $p = 0.0002$, day 1 vs day 2, $p = 0.0148$ and day 1 vs day 3, $p = 0.0001$; [B] Experimental timeline. [C] OT: RM one-way ANOVA, Dunnett; average trials: $p = 0.0046$, day 1 vs day 3, $p = 0.0057$; day 1 vs day 4, $p = 0.0057$ and day 1 vs day 5, $p = 0.0036$; RT: $p = 0.0022$, day 1 vs day 3, $p = 0.0027$; day 1 vs day 4, $p = 0.0011$ and day 1 vs day 5, $p = 0.0275$; ITI: $p < 0.0001$, day 1 vs day 3, $p = 0.0004$; day 1 vs day 4, $p < 0.0001$ and day 1 vs day 5, $p = 0.0002$; correct: $p = 0.0025$, day 1 vs day 2, $p = 0.0464$; day 1 vs day 3, $p = 0.0043$; day 1 vs day 4, $p = 0.0042$ and day 1 vs day 5, $p = 0.0013$. [D] Retention of the test: average trials: $p = 0.0058$; baseline vs 4 months, $p = 0.0042$; RT: $p < 0.0001$, baseline vs 6 d, $p = 0.0063$; baseline vs 4 months, $p < 0.0001$; ITI: $p < 0.0001$, baseline vs 27 d, $p = 0.0093$, baseline vs 3 months, $p = 0.0009$, baseline vs 4 months, $p = 0.0252$; correct: $p = 0.2290$

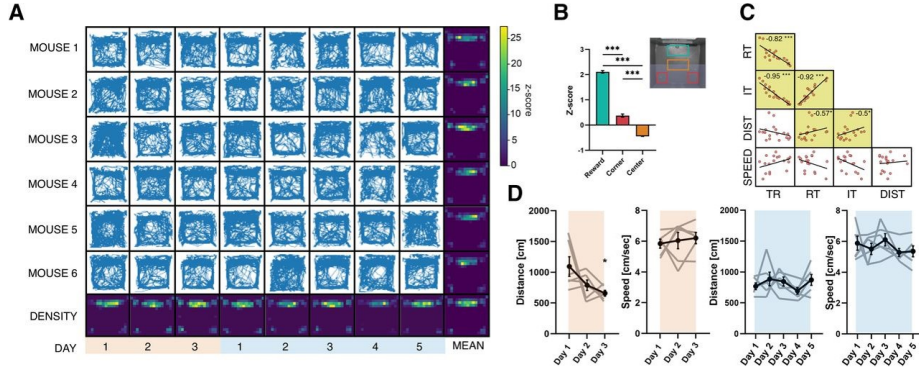


Figure 4.12: *[A]* Matrix of tracking traces of all animals per all days, with marginal heatmaps, showing spots of exploration significantly different from chance. Average heatmaps per each animal and per each day are presented in the last column and in the last row, respectively. *[B]* Relative exploration: RM one-way ANOVA, Dunnett's multiple comparisons post hoc: $p > 0.0001$, corner vs center, $p > 0.0001$; corner vs reward, $p > 0.0001$, *[C]*, Correlation analysis. Spearman's correlation: TR vs RT: $r = -0.8223$ (95% CI, -0.9335 to -0.5669); $R^2 = 0.6761$; ITI vs RT: $r = 0.9209$ (95% CI, 0.7910 to 0.9714); $R^2 = 0.8480$; DIST vs RT: $r = 0.5666$ (95% CI, 0.1208 to 0.8222); $R^2 = 0.3210$; DIST vs ITI: $r = 0.4972$ (95% CI, 0.02450 to 0.7882); $R^2 = 0.2472$; *[D]* Velocity and distance traveled; tracking distance: One-way ANOVA, Holm-Sidak's post hoc: $p = 0.03$; day 1 vs day 3, $p = 0.02$

optimization of the psychomotor sequence in realizing the task rather than to a general increase of velocity of the animal (Mazziotti et al. 2020a).

4.2.2 Evaluation of CDKL5 mice performance in learning task

In order to evaluate the learning abilities of CDKL5 mice, I performed OC protocol in fully symptomatic male KO mice and WTm littermates controls and subsequently in female HET mice compared to WTf littermates.

CDKL5 null mice performance during shaping indicate a hyperactive phenotype

On day 3 of the shaping phase (Fig 4.13 [A]) CDKL5 null mice perform a higher average number of trials; looking at the individual days, the two groups start from the same performance and then go on to differentiate over time.

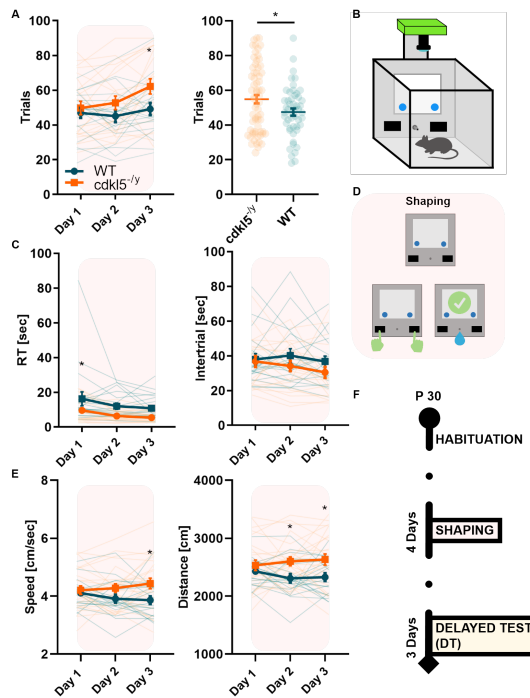


Figure 4.13: [A] Single day trials: two-way ANOVA, time < 0.05 , genotype < 0.05 ; post hoc Sidak at Day 3 < 0.05 ; average trials: P-value < 0.05 , Unpaired T-Test; [B] RT: two-way ANOVA, < 0.005 , genotype < 0.005 ; post hoc Sidak at Day 1 < 0.05 ; ITI: no difference; [C] Locomotor activity: speed: two-way ANOVA, < 0.05 ; post hoc Sidak at Day 3 < 0.05 ; distance: two-way ANOVA, < 0.05 ; post hoc Sidak at Day 3 < 0.05

Moreover, for the first day of shaping, KO mice show reduced reaction time,

but no difference in the intertrial interval (Fig. 4.13 [B]). Finally, from tracking analysis (Fig. 4.13 [C]), I assess movement patterns during learning tasks, specifically, I measured speed and distance parameters. I found a difference at day 3 in speed parameter, KO resulted faster than WT, furthermore CDKL5 null mice covered a greater distance at day 3 compared to WT. These results indicate that CDKL5 null mice have no deficit in learning abilities, but these suggest a hyperactive and impulsivity phenotype that could lead to arousal alteration.

The same performance is also visible in CDKL5 female mice

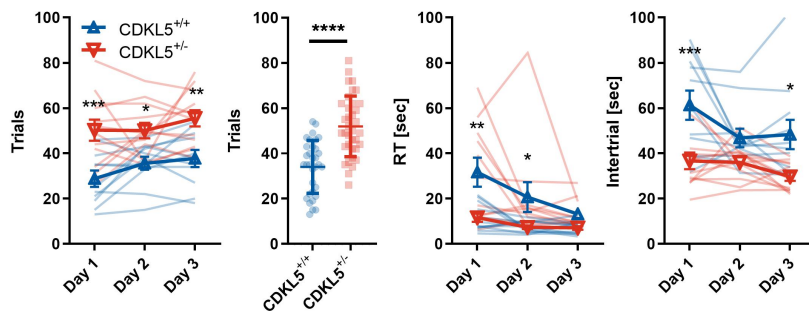


Figure 4.14: [A] Single day trials: two-way ANOVA, genotype <0.001 ; post hoc Sidak at Day 1 <0.001 , Day 2 <0.05 , Day 3 <0.01 ; average trials: P-value <0.0001 , Unpaired T-Test; RT: two-way ANOVA, <0.05 , genotype <0.05 ; post hoc Sidak at Day 1 <0.01 , Day 2 <0.05 ; ITI: two-way ANOVA, time <0.05 , genotype <0.01 ; post hoc Sidak at Day 1 <0.001 , Day 3 <0.05 .

In order to evaluate the learning ability in female mice, I evaluated HET mice, compared to WT littermates, in the shaping test. As reported in 4.14 [A] HET mice performed more trials, less RT and Intertrial interval, suggesting the same hyperactivity phenotype detected in KO mice.

The delayed tasks show a low level of cognitive flexibility, perseverative behaviour and hyperactivity

To better understand the hyperactive behavior that CDKL5 null mice exhibit during shaping, I introduce the delayed test (DT). Interestingly I found an inversion of the curve in the number of trials and intertrial: (4.15 [A]): CDKL5 null mice take longer to acquire the new strategy. Indeed, I found a difference between groups in the number of trials on the first and second day of DT that disappeared the last day. Even for the intertrial, I found the same trend with a significant difference on the first and second days. Moreover, I analyzed the perseveration: that parameter measures every time mice performed the previous strategy instead of the new one. CDKL5 null mice continue to implement the same strategy of shaping for the first two days of DT as compared to WT littermate.

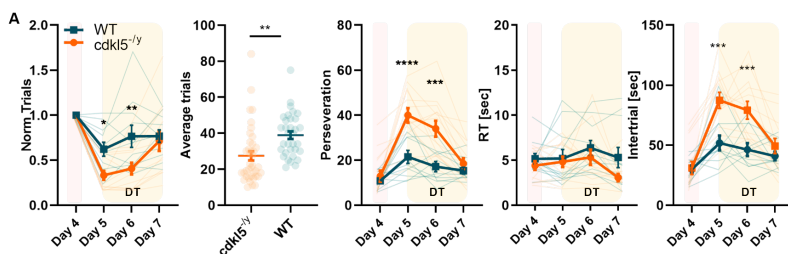


Figure 4.15: Normalized trials: two-way ANOVA, interaction time x genotype <0.01 , post hoc Sidak at Day 5 <0.05 and Day 6 <0.01 ; average trials: P-value <0.01 , T-Test; Perseveration index: two-way ANOVA, interaction time x genotype <0.001 , post hoc Sidak at Day 5 <0.0001 and Day 6 $= 0.0001$; RT: no difference; ITI: two-way ANOVA, interaction time x genotype <0.01 , post hoc Sidak at Day 5 <0.001 and Day 6 <0.001

These results suggest a lack of cognitive flexibility and impulsive behavior. Finally, no difference in reaction time was identified, confirming that the decrease

of trials and the increase of intertrial were not attributable to a lack of interest in the task. Taken together, those results indicate that the lack of CDKL5 leads to impulsivity, hyperactivity, perseverative behavior, and poor cognitive flexibility suggesting a defect in arousal.

4.2.3 DREADD inhibition of PFC elicits perseverative response and hyperactivity

As reported in literature (Le Merre et al. 2021) the prefrontal cortex (PFC) plays an important rule in any aspect of higher-order cognitive functions, as rule representation, context discrimination (Rikhye et al. 2018; Schmitt et al. 2017), action planning (Inagaki et al. 2019), and working memory (Bolkan et al. 2017; Kim et al. 2016). Moreover, in recent studies (Rikhye et al. 2018; Schmitt et al. 2017), the authors investigate the rule of PFC connection to thalamus in cognitive flexibility. To investigate the rule of PFC in AC test, I injected C57B-6J mice with inhibitory DREADD in PFC.

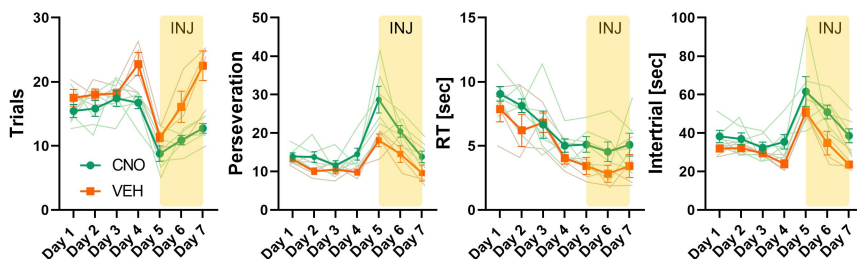


Figure 4.16: Normalized trials: two-way ANOVA, interaction time x genotype: 0.0058

From preliminary data, as reported in fig. 4.16, DREADD activation with CNO administration during DT, seems to disrupt the ability to quickly shift to

the new task, eliciting impulsive behavior and perseverations. Nevertheless, the number of subjects tested is not enough to have meaningful data; however, an encouraging curve trend is visible, supported by the statistical significance of the interaction in the number of trials. These results suggest that PFC plays a crucial role during DT, and perturbation of this area leads to a lack of cognitive flexibility.

Chapter 5

Discussion

Neurodevelopmental disorders (NDDs) are a heterogeneous group of conditions involving some form of disruption to brain development, occurring during infancy, and in most cases, having a chronic progression over a lifetime.

This thesis focused on a subset of monogenic NDDs, particularly CDKL5 deficiency disorders (CDD), Creatine transporter deficiency (CTD) syndrome, Krabbe disease, and Angelman syndrome. These conditions have a robust, known genetic origin and a devastating impact on patients and their families. I grouped these different NDDs in my project because recent studies indicate that common physiological mechanisms, related to brain energetics, might concur to the pathophysiology of these disorders, with upregulation of respiratory chain complexes, elevated ROS production, decrease in ATP production, increased synthesis of TCA cycle, and glycolytic enzymes (Oyarzábal et al. 2021).

Unfortunately, there is no such cure for these disorders; medication comprises symptomatic and palliative drugs able, in some mild cases, to partially reduce

the most impacting symptoms, like seizures and behavioral problems. However, these therapeutic strategies do not represent a cure, and patients require life-long care, resulting in a large impact on the quality of life of both patients and caregivers, as well as a burden on the healthcare system.

Animal models for these disorders are available and recapitulate different critical features of human conditions, resulting in good testbeds for evaluating the pathophysiology of the disease and potential therapeutic approaches at the preclinical level. However, there remains a pressing need for unbiased quantitative biomarkers of high translational value to non-invasively and quantitatively monitor brain function (Mazziotti et al. 2017). This is an important problem, because the phenotypic observation of behavioral endpoints might be highly prone to subjective bias, and the use of objective readouts for preclinical and clinical trials is crucial to properly evaluate the therapeutic response to new drugs. In 2000 the U.S. National Institutes of Health (NIH) convened a Biomarkers Definitions Working Group (NIH Definitions Working Group, 2000; Biomarkers Definitions Working Group, 2001) that defined a biomarker as “a characteristic that is objectively measured and evaluated as an indication of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” Clinical biomarkers have the advantages of being simpler and less expensive to measure than final clinical endpoints and they can be analyzed repeatedly and over a shorter period of time. The use of biomarkers allows clinical trials to be performed with fewer subjects than might otherwise be possible (Aronson and Ferner 2017). Objective biomarkers have also the potential to be helpful in the management of patients, allowing the classification of disease severity and monitoring response to treatments (Aronson and Ferner 2017).

In order to establish a novel biomarker for NDDs, I used transcranial intrinsic

optical signal (IOS) imaging to investigate visual cortical function in CDD, CTD, Krabbe and Angelman syndrome. Moreover, I recorded Visual Evoked Potentials (VEPs) in CDD and CTD. In recent years, the visual cortex has become an important model to assess cortical processing in mouse models of neurodevelopmental disorders (Durand et al. 2012; Begenisic et al. 2015; Boggio et al. 2016). For instance, the use of visually evoked responses has been suggested as a relatively common quantitative method to directly compare cortical function in Rett patients and mouse models carrying *mecp2* mutations (LeBlanc et al. 2015; Duran-Trio et al. 2021). VEPs have also been used to reveal alterations in West Syndrome patients, EIEE1 (OMIM ref: #308350), and in EIEE3 (OMIM ref: #609304), supporting the translational and clinical applicability of this technique even in presence of seizures or hyperexcitability (de Freitas Dotto; Molinari). Accordingly, monitoring of visual function proved to be a precise tool, highlighting alterations in the intrinsic spectral properties of oxygenated and deoxygenated hemoglobin in the primary visual cortex in each of the strains investigated. In particular, in *CDKL5* mice, the results showed that cortical responses are dramatically reduced in amplitude already at P27-P28, as assessed both with IOS and VEPs (Mazziotti et al. 2017), suggesting an early disruption of cortical amplitude, which begins during critical period. Moreover, this reduction is significantly correlated with decreased response amplitude, abnormal contrast response function, and impaired visual acuity in adult mutants (Mazziotti et al. 2017). Importantly, these alterations were detected both in male mice, with no expression of *cdkl5*, and in heterozygous female mutants, with mosaic expression of the remaining *CDKL5* allele (Mazziotti et al. 2017). Sensitivity analysis revealed that visual responses were able to correctly classify mice in mutant or wild-type with more than 93% accuracy and 92% sensitivity proving the

robustness of this technique in phenotype assessment (Mazziotti et al. 2017).

However, a dissection of the abnormalities in the visual circuitry responsible for the origin of VEP alterations remains elusive. Indeed, damage anywhere along the visual path may affect the signal in the cortex; this thesis provides evidence supporting a cortical origin of the visual response deficit occurring in a conditional mouse model of CDD. Indeed, visual cortical neuronal morphology in the visual cortex was strongly affected by CDKL5 mutation, and deletion of CDKL5 from excitatory cells of the visual cortex was sufficient to elicit a significant impairment of visual responses (Lupori et al. 2019).

As reported in my results, the onset of visual response impairment in mice take place during a period of intense synaptogenesis and synaptic plasticity, in agreement with the proposed role of CDKL5 in synaptic organization (Della Sala et al. 2014; Zhang et al. 2021; Zhu and Xiong 2019). Previous studies performed in CDKL5 mice reported abnormal turnover and density of dendritic spines, and reduced expression of proteins involved in excitatory synaptic structure in different brain areas, including sensory cortices (Della Sala et al. 2014; Amendola et al. 2014; Trazzi et al. 2018). In particular, a morphological analysis focused on the developing visual cortex showed reduced staining for the activity-dependent protein c-fos and for key components of the excitatory postsynaptic protein assembly together with increased numbers and connectivity of parvalbumin-positive inhibitory interneurons (Pizzo et al. 2016). The possibility that CDKL5 acts both on excitatory and inhibitory neurons is strengthened by previous work showing distinct but overlapping phenotypes in mice with CDKL5 deletion selectively from excitatory or inhibitory neurons (Amendola et al. 2014). Therefore, a defective maturation of excitatory and inhibitory circuits could contribute to the impaired visual cortical responses (both in IOS and VEP analysis) observed

here.

By contrast, analyzing visual amplitude in CTD, I found a strikingly specific increase in cortical IOS responses in CrT-/y mice. It has been previously reported a specific impairment of inhibitory synapses in the CTD murine model with no alteration of glutamatergic neurotransmission (Baroncelli et al. 2016b), suggesting that functional deficits result from relatively subtle changes in synaptic connectivity. However, the normal visually evoked potential amplitude recorded in electrophysiological experiments using CRT-/y mice suggests that the increased IOS responses originate from a disruption of neurovascular coupling. In light of the ubiquitous expression of Slc6a8 gene in the brain at the cellular and regional level (C. S. W. Mak et al. 2009), the normal visually evoked potential amplitude recorded in the visual cortex of CrT-/y mice suggests that glial and vascular cells might be the cellular basis of altered IOS. Accordingly, the forced metabolic phenotype and the resultant increase in cellular oxidative stress observed in the brain of CrT-/y animals (Giusti et al. 2019) could dynamically upregulate the cerebral blood flow stimulating vasodilation (Watts et al., 2018). Random Forest machine learning algorithm for data classification shows an ability of classifying animal genotypes at single subject level with high accuracy and sensitivity (>85%) and to measure disease progression (Mazziotti et al. 2020a). This tool may be of high translational value for future preclinical studies of creatine transporter deficiency, and also more broadly for neurodevelopmental disorders presenting with cognitive dysfunction, autistic-like features and brain hyperexcitability. Further confirmation that IOS is a valid functional biomarker arises from the results of the administration of cyclocreatine (cCr) treatment in CTD mice. Indeed, I monitored IOS amplitude responses, longitudinally in CTD during cCr administration, finding that this treatment can partially correct

cortical hemodynamic responses caused by Cr depletion (Cacciante et al. 2020b).

Interestingly, I found the same increase in IOS amplitude responses, detected in CTD mice, in twitcher and UBE3A animals. These results, together with the hypothesis of the disruption of brain bioenergetics in these NDDs, suggest that an alteration of neurovascular coupling might occur in these disorders as well. However, there is a lack of information regarding VEPs in vivo in these pathologies, that require further investigations. Taken together, these data reinforce the concept that, optical imaging technique, such as IOS imaging and functional near-infrared spectroscopy (fNIRS, (Lloyd-Fox et al. 2010; Vanderwert and Nelson 2014)) might allow clinicians to optimize the follow-up of patients and to monitor the efficacy of potential therapeutic strategies on brain activity. fNIRS was studied in humans as a non-invasive measure of cerebral hemodynamics, which is similar to the main components of IOS (Tsystarev 2012). Indeed, it allows quantifying oxygen consumption in different regions of the cerebral cortex, providing an indirect measure of neuronal activity (Lloyd-Fox et al. 2010; Vanderwert and Nelson 2014). This blood-oxygen-level-dependent (BOLD) signal is similar to that detected with functional MRI (fMRI; (Raichle and Mintun 2006)). However, fNIRS has the advantage of being totally non-invasive, low-cost, portable, noiseless, endowed with high experimental flexibility and no setting constraints. Moreover, fNIRS is more tolerant to motion artifacts than fMRI, and the development of robust methods for motion detection/correction allowed to avoid sedation in children (Vanderwert and Nelson 2014; Di Lorenzo et al. 2019). These methodological strengths provide fNIRS with a high ecological value for investigating neural circuits either in typically developing or clinically relevant populations. Growing evidence suggests that fNIRS might be a candidate biomarker for several neuropsychiatric disorders, including ASD (Y. Li and Yu

2016; Yasumura et al. 2019; Yang et al. 2019). In particular, functional network efficiency (Y. Li and Yu 2016), weighted separability of NIRS signals (Yanagisawa et al. 2016), multi-layer neural networks and sample entropy of spontaneous hemodynamic fluctuations (Xu et al. 2020; Xu et al. 2021) have been proposed as auxiliary diagnosis indexes for ASD. However, all these approaches require complex algorithms to extract high-level features from the fNIRS raw data, while fitness, applicability and translational value of biomarkers greatly depend on their ease of use. In this framework, the analysis of visual phenotype is quick, easy and optimizes the compliance of young subjects (Mazziotti et al. 2021). The underlying pathological alteration, such as metabolic disruptions, brain hyperexcitability, a defective maturation of excitatory and inhibitory circuits and other, that might explain the physiological alterations detected in NDDs, could also be responsible for the behavioral alterations of these patients. These manifestations result in a wide range of cognitive and behavioral symptoms that in some cases, are an element of enormous stress for caregivers. These manifestations include autistic features, as stereotypes, lack of languages, hyperactivity, psychopathological symptoms (e.g. anxiety, depressions, sleep disturbances and other) and self-directed and heterodirected aggressive behaviors. The assessment of such manifestations represents a primary point for physicians, psychologists and researchers, both in clinical and research analysis, in order to better understand these manifestations and set up an appropriate treatment plan. In the clinical setting there are numerous tests aimed at evaluating and measuring these behaviors that can also be applied in clinical research. Mice models of these disorders recapitulate most of the symptoms detected in humans. However, the classical paradigms used in neuroscience are subjected to different biases that reduce the reliability and accuracy of these measures. There is a

pressing need for behavioral biomarkers capable of answering these questions; for these reasons, I implemented a fully 3D printable device that performs OC on freely moving mice while tracking the animal position in real-time. I successfully trained mice, whose performance, detected by this system in three sessions per day (3.97 ± 0.11 trial/min with an accuracy of $84.1 \pm 1.7\%$), are comparable with normative values detected in C57BL/6J and measured on an analogous two-alternative forced choice task performed once daily (Malkki et al. 2010). Moreover, the OC chamber includes real-time tracking of the mouse position, a feature that could be used as a second phenotyping measure of anxiety or stereotyped behaviors. Additionally, it allows the analysis of other aspects of behavior, such as inhibitory control (Munakata et al. 2011). For example, by increasing the time required to trigger a trial, it is possible to measure impulsivity or reproduce neuropsychological tests used in humans. It is also possible to couple the procedure with physiological recordings in freely moving conditions such as imaging techniques (e.g., fiber photometry) and electrophysiology. Thanks to the complete automaticity of the test and the ability to accurately detect parameters, its results as a robust behavioral tool, applicable to monitor longitudinally the effectiveness of treatments and washout effects in preclinical, and possibly clinical, studies in NDDs.

Applying this tool to CDKL5 mice, I found both in males and females, shorter reaction times, more trials per session, and hyperlocomotion suggesting a phenotype characterized by impulsivity and hyperactivity. To further investigate this phenotype, I moved to evaluate inhibitory control by increasing the time required to trigger a trial. As a result, CDKL5 KO mice failed to acquire the new strategy, showing perseverative errors. Errors of perseveration arise from a deficit in cognitive flexibility (CF); CF is the ability to shift rapidly from a

previously learned action-reward association to a new one when the rules of a task change. The brain region most critically involved in CF is the prefrontal cortex (PFC). Specifically, comprehensive literature has demonstrated that the functional integrity of PFC is essential for CF in rodents (Ragozzino et al. 2003; Stefani et al. 2003; Stefani and Moghaddam 2010), primates (Dias et al. 1996; Wallis et al. 2001) and humans (Konishi et al. 1998; Nagano-Saito et al. 2008). For these reasons, I'm now moving to investigate the rule of PFC in OC chamber, inhibiting the action of the foregoing cortex by the injection of chemogenetics drugs, during the evaluation of inhibitory control, in C57BL/6J mice. Injected mice fail to acquire the new strategy, showing perseverative errors, these results pinpoint the PFC as a potential neural substrate; moreover, these data recapitulate the CDKL5 deficit in CF, suggesting the destruction of some circuits in the PRF of KO mice.

In summary, this thesis provides evidence that supports the use of the visual system as a translational and non-invasive biomarker for the investigation of brain function in four different monogenic NDDs. Moreover, I validated this biomarker in preclinical studies on CTD, proving its ability to detect changes in neurovascular coupling after cCr administration. Furthermore, I introduce and validate OC chamber, as an automated tool for appetitive conditioning in mice, detecting hyperlocomotion and impulsive behavior in CDKL5 KO mice. Despite the availability of reliable tools for the diagnosis, such as biochemical and genetic analyses, and the behavioral assessments, as the classical paradigms used to evaluate cognitions, of these disorders; the biomarkers discovered here could lead to improvements in clinical settings and research. Specifically, the evaluations of these biomarkers will allow clinicians in the assessment and follow-up of patients accurately identifying any alterations of brain circuits during

the progression of the disorder. Moreover, combining imaging tools, such as fiber photometry and miniscope, with behavioral testing provides a very reliable protocol to longitudinally monitor the efficacy of potential therapeutic strategies in preclinical, and possibly clinical, studies.

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