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Digital Ventilated Cages (DVC®) as a tool for the study of Machado-Joseph Disease: Influence of physical exercise on established motor dysfunction in a transgenic mouse model of MJD

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Resumo

DVC® (Digital Ventilated Cage), é um sistema de monitorização automático de gaiolas, capaz de detetar continuamente a atividade espontânea de animais que ocorre na gaiola 24 horas por dia, 7 dias por semana. Esta monitorização permite um estudo não intrusivo, proporcionando um melhor bem-estar e uma potencial melhoria na reprodutibilidade experimental. Neste estudo, avaliamos a influência do exercício físico como estratégia terapêutica para melhorar a disfunção motora em murganhos, um modelo de animal da doença de Machado-Joseph (DMJ), o CMVMJD135. A DMJ é a ataxia autossómica dominante mais comum em todo o mundo, sendo uma doença neurodegenerativa causada por uma expansão da repetição do tripleto CAG dentro do gene da Ataxina-3, conduzindo ao aparecimento de diversos sintomas, maioritariamente afetando a coordenação motora. A variabilidade clínica do fenótipo da doença, bem como a idade de início, dependem do tamanho da repetição expandida do tripleto de CAG. O murganho CMVMJD135 mimetiza a doença humana tanto a nível comportamental como patológico. Neste estudo, os animais foram divididos em 4 grupos (Estirpe-Selvagem (WT); Transgénicos (TG); WT com roda (WTRW); Transgénicos com roda (TGRW)), com uma distribuição de 2 animais por gaiola. O DVC® recolheu automaticamente dados sobre atividade espontânea na gaiola e exercícios físicos voluntários na Roda. Além disso, foram realizados outros testes relacionados com a coordenação motora, que estão padronizados e estabelecidos no laboratório para caracterizar o início e progressão da doença em murganhos DMJ, permitindo assim avaliar a influência da atividade espontânea dentro da gaiola e o desempenho na Roda de cada grupo. A análise da atividade geral dos animais durante o período noturno mostrou que os murganhos TG tiveram uma atividade significativamente menor quando comparados aos seus companheiros de ninhada WT. Adicionalmente, os murganhos TG com acesso a roda foram mais ativos do que os murganhos TG sem Roda. Quanto ao exercício praticado na Roda, o grupo WT apresentou valores muito superiores aos TG. Em relação aos testes de comportamento adicionais, os resultados gerais mostraram uma tendência geral de os murganhos TG com acesso à Roda apresentarem um melhor desempenho quando comparados aos murganhos TG sem acesso à Roda. No geral, estes resultados sugerem que o sistema DVC® é capaz de detetar o fenótipo dos murganhos DMJ sem a interferência do experimentador e a presença de Rodas nas gaiolas parece melhorar o seu fenótipo motor. Estes resultados promissores apontam para uma caracterização adicional e mais extensa do exercício físico neste modelo de doença. Adicionalmente, este sistema

automatizado pode ser de grande importância para outros modelos de doenças neurodegenerativas, sendo uma ferramenta complementar aos testes de comportamento animal comumente utilizados por laboratórios de todo o mundo.

Palavras-chave

Doença Machado-Joseph; Ataxia; Exercício físico; Terapia; Comportamento; Análise automática comportamental; DVC®

Resumo Alargado

DVC® (Digital Ventilated Cage), é um sistema de monitorização automático de gaiolas, capaz de detetar continuamente a atividade espontânea de animais que ocorre na gaiola 24 horas por dia, 7 dias por semana. Esta monitorização permite um estudo não intrusivo, proporcionando um melhor bem-estar e uma potencial melhoria na reprodutibilidade experimental.

Neste estudo, avaliamos a influência do exercício físico como estratégia terapêutica para melhorar a disfunção motora num modelo animal da doença de Machado-Joseph (DMJ), o CMVMJD135. Para além disso, tínhamos também como objetivo testar a capacidade do sistema automatizado DVC® para detetar a progressão da doença no modelo CMVMJD135.

A DMJ é a ataxia autossómica dominante mais comum em todo o mundo, sendo uma doença neurodegenerativa causada por uma expansão da repetição do tripleto CAG dentro do gene da Ataxina-3, conduzindo ao aparecimento de diversos sintomas, maioritariamente afetando a coordenação motora. A variabilidade clínica do fenótipo da doença, bem como a idade de início, dependem do tamanho da repetição expandida do tripleto de CAG. O murganho CMVMJD135 mimetiza a doença humana tanto a nível comportamental como patológico.

Neste estudo, os animais foram divididos em 4 grupos (Estirpe-Selvagem (WT); Transgénicos (TG); WT com roda (WTRW); Transgénicos com roda (TGRW)), com uma distribuição de 2 animais por gaiola. O DVC® recolheu automaticamente dados sobre atividade espontânea na gaiola e os exercícios físicos voluntários na Roda de corrida. Além disso, foram realizados outros testes relacionados com a coordenação motora, que estão padronizados e estabelecidos no laboratório para caracterizar o início e progressão da doença em murganhos DMJ, permitindo assim avaliar a influência da atividade espontânea dentro da gaiola e o desempenho na Roda de cada grupo.

A análise da atividade geral dos animais durante o período noturno mostrou que os murganhos TG tiveram uma atividade significativamente menor quando comparados aos seus companheiros de ninhada WT. Adicionalmente, os murganhos TG com acesso a roda foram mais ativos do que os murganhos TG sem Roda. Quanto ao exercício praticado na Roda, o grupo WT apresentou uma média de 1,5 quilómetros diários

percorridos e os TG cerca de 0,8 quilómetros. Como seria de esperar, a distância média diária percorrida pelos WT é muito superior aos TG. Sendo o DVC® uma tecnologia ainda em fase de estudos e de desenvolvimento, surgiram pequenos problemas técnicos relacionados com as primeiras versões das Rodas de corridas. Como a resolução desses problemas fez também parte do presente estudo, o desenho experimental teve que passar por várias adaptações, e só foi possível iniciar o estudo numa fase tardia da doença. Assim é de salientar que, embora os dados apresentados neste trabalho compreendam idades entre as 30 e 39 semanas, os animais tiveram acesso a versões não finais, anteriores à estudada, a partir das 6 semanas de idade. Dito isso, é importante realçar que os animais não iniciaram a prática de exercícios físicos tardiamente, mas sim desde as primeiras semanas de idade.

Em relação aos testes de comportamento adicionais, os resultados gerais mostraram uma tendência geral de os murganhos TG com acesso à Roda apresentarem um melhor desempenho quando comparados aos murganhos TG sem acesso à Roda. É possível que os efeitos benéficos observados pela presença da Roda neste estudo estejam correlacionados com o facto de os animais terem tido acesso às diferentes versões da Roda, embora que estas não correspondessem a todas as exigências, no entanto, terão proporcionado algum grau de exercício físico voluntário aos animais.

No geral, estes resultados sugerem que o sistema DVC® é capaz de detetar o fenótipo dos murganhos DMJ sem a interferência do experimentador e a presença de Rodas nas gaiolas parece melhorar o seu fenótipo motor. Estes resultados promissores apontam para uma caracterização adicional e mais extensa do exercício físico neste modelo de doença. Adicionalmente, este sistema automatizado pode ser de grande importância para outros modelos de doenças neurodegenerativas, sendo uma ferramenta complementar aos testes de comportamento animal comumente utilizados por laboratórios de todo o mundo.

Abstract

DVC® (Digital Ventilated Cage), a home-cage rack monitoring system is capable of continuously detecting spontaneous animal activity occurring in their home cage 24/7. This monitoring allows a non-intrusive study, providing a better welfare and a potential improvement in experimental reproducibility. In this study, we evaluated the influence of physical exercise as a therapeutic strategy to improve motor dysfunction in a mouse model of Machado–Joseph Disease (MJD), the CMVMJD135. MJD is the most common autosomal dominant ataxia worldwide, a neurodegenerative disorder caused by a CAG repeat expansion within the Ataxin-3 gene, causing mostly motor symptoms. The clinical variability of the disease phenotype as well as the age of onset depend on the length of the expanded repeat. The CMVMJD135 resembles the human disorder both at the behavioral and pathological levels. In this study, the mice were divided into 4 groups (Wild-Type (WT); Transgenic (TG); Wild-Type with Running Wheel (WTRW); Transgenic with Running Wheel (TGRW)), with a distribution of 2 animals per cage. The DVC® automatically collected data on spontaneous activity in the cage and voluntary physical exercise on the Running wheel (RW). Additionally, other motor-related tests were performed, which are usually the standard for characterizing the disease establishment and progression in MJD mouse models, to understand the influence of spontaneous activity within the cage and the performance in the RW by each group. The analysis of the overall animals' activity during the night period showed that TG mice had significant less activity when compared to their WT-littermates. Additionally, TG mice with access to RW where more active than TG mice without RW. As for the exercise practiced on the RW, the WT group showed much higher values compared to TG. Regarding the additional behavior tests, the overall results showed a tendency for the TG mice with access to the Running Wheel to present a better performance when compared to TG without RW. Overall, these results suggest that the DVC® system is able to detect the phenotype of the MJD mice without the experimenter interference and the presence of running wheels in the cages seem to improve their motor phenotype. These promising results point to further and more extensive characterization of physical exercise in this mouse model. Furthermore, this automated system may be of great importance to other models of disease, being a complementary tool to animal behavior testing.

Keywords

Machado-Joseph disease; Spinocerebellar ataxia; Physical exercise; Therapy; Behavior; Automated Behavioral Analysis; DVC®

Index

List of figures

List of tables

List of acronyms

ATXN3	Ataxin-3 gene
CAG	Cytosine Adenine Guanine
CMV	Cytomegalovirus
CMVMJD135	Transgenic mouse model
CNS	Central Nervous System
DGAV	Direção Geral de Alimentação e Veterinária
DNA	Desoxyribonucleic Acid
DVC®	Digital Ventilated Cages
EGF	Epidermal Growth Factor
FELASA	Federation of European Laboratory Animal Science Associations
ICVS	Life and Health Science Research Institute
IVC	Individually Ventilated cages
MJD	Machado-Joseph Disease
NIIs	Nuclear Intracellular Inclusions
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
PVC	Polyvinyl Chloride
RNA	Ribonucleic Acid
RW	Running Wheel
SCA3	Spinocerebellar Ataxia type 3
TAE	Tris Acetate-EDTA
TG	Transgenic
TGRW	Transgenic with access to Running Wheel
UV	Ultra Violet
WT	Wild-Type
WTRW	Wild-Type with access to Running Wheel

List of communications

Poster presentation:

- NeRD Retreat - Lockdown Version 2020, Braga, Dezembro 2020: **A. Santos**, D. Monteiro-Fernandes, S. Duarte-Silva, MJ. Castelhano-Carlos, Digital Ventilated Cages (DVC®) as tool for the study of Machado-Joseph Disease: Influence of physical exercise on established motor dysfunction in a transgenic mouse model of MJD. (The poster can be found in the attachments).

Chapter 1 – Introduction

Machado-Joseph disease: an historical overview

Machado Joseph disease (MJD) emerged in the 70s and soon aroused enormous interest in the scientific community. The first cases occurred in 3 Azorean families residing in the United States. The initial medical and scientific interest may have been related to the influence of the Luso-American communities. The moment when this disease appeared was coincident with the moment in knowledge of genetic diseases of the nervous system started to progress until what is known today. Thus, with all the interest from the scientific community, studying the families and populations affected, as well as the interest from the media and from social and political organizations (essentially in the United States), led to the description of the families initially affected, registration and further study of the disease, contributing for the identification of the common genetic marker found to be affected in families, with MJD worldwide [1]–[3].

For the knowledge of this new disease, some problems had to be overcome, such as, the identification of the first affected families, or the uniqueness of the described pathologies; however, the biggest controversy was the designation to be given to the disease. Between 1972 and 1977, MJD was identified in four families, reported as four distinct entities.

Below, listed in chronological order, are the different designations proposed for MJD:

1972 - Machado's disease, used by Nakano and Dawson in the original article on the family of the same name [4].

1972 - Nigro-spinodental degeneration with nuclear ophthalmoplegia, according to the results of the first autopsy described by Woods and Schaumburg [5].

1976 - Autosomal dominant striatonic degeneration, proposed by Rosenberg, Nyhan and Bay [6].

1977 - The Azorean disease of the nervous system, suggested by Romanul and Fowler when describing the fourth Azorean family and the proposal for unification [7].

There were some inconsistencies on the topic, namely the neurological disease in the Azores and the MJD of the Azores by Fowler in 1978 [8]. The latter designation was accepted by American neurologists who saw, in this case, a new disease based on affected families of Azorean origin. This designation lead to a stigma of a certain population that had the United States as an emigration destination of their ancestors and, on the other hand, an incorrect designation since other families were found in the United States and in the rest of the world [2].

> **1977** – Joseph's disease, used by Rosenberg when he started to accept the arguments raised against the previous neuropathological designation. With the same name, the International Joseph Diseases Foundation was created (note that it was in the plural, "diseases", and not in the singular, suggesting some uncertainties about the uniqueness of the families described) [3].

Still, in 1975, Coutinho and Andrade proposed that the diseases mentioned above were simply variations of the same clinical disorder (Coutinho and Andrade, 1978) [3]. Thus, they determined that it should be called "Machado-Joseph disease", a unique disease characterized by enormous unusual clinical variability [9].

> **1980 -** Machado-Joseph disease, first proposed by Coutinho and Sequeiros at the Lisbon Symposium on "autosomal system diseases dominant in populations of Portuguese origin" [10].

After many years and many controversies, attempting several designations based on ethnic, neuropathological, genetic, and other criteria, the name Machado-Joseph disease was officially adopted.

Machado-Joseph disease or Spinocerebellar Ataxia type 3 (MJD/SCA3) general overview

Machado-Joseph disease (MJD), also known as Spinocerebellar Ataxia type 3 (SCA3), is the most common autosomal dominant ataxia worldwide [11], [12].

MJD is spread worldwide, being the highest prevalence of 1:140 found on the small Azorean island of Flores [13]. The identification of MJD cases, with diverse ethnic origins, was made possible by researchers using DNA-based studies. The highest relative frequency is registered in Portugal (49%) among spinocerebellar ataxias. China follows (49%), and then, in descending order of percentage is Brazil (44%), Holland (44%), Japan (43%) and Germany (42%). The lowest relative frequencies are recorded

in France (33%), the United States (21%) and Australia (12%). However, there are countries where the disease is very rare, such as the United Kingdom (5%), India (3%) and Italy (1%) [12], [14]–[16].

Clinical definition

It is difficult to accurately establish the age of onset of MJD, but patients suffer from a progressive neurodegenerative disorder, frequently appearing between the ages of 20 and 50, in which the intellect is preserved [3], [17], [18].

However, the average survival time for patients is about 21 years. This life expectancy varies depending on the degree of severity of the disease, and in the most severe form the survival time can go up to 30 years, whereas in the lighter form of the disease patients can have a common life. Importantly, the cognitive function is preserved, which is a fundamental characteristic of MJD in the differential diagnosis between the various spinocerebellar ataxias.

This disease can be characterized by lack of motor coordination and gait ataxia, difficulty in speech and food intake, spasticity (associated with dystonia presented by some patients, with muscle stiffness or contractions that cause body and limb torsion, abnormal body posture), frequent urination and changes in vision. Some patients also have peripheral neuropathy, loss of muscle strength or Parkinsonism [19], [20].

Regarding the onset of the disease and its severity, it has been shown that the onset and the severity of the disease are interconnected, and most serious and rapid forms of disease have been registered at an earlier onset [21], [22]. As a progressive disease, MJD is characterized by the accumulation of symptoms that worsen over time.

Given the different clinical manifestations of SCA3, four clinical sub-phenotypes were defined [3], [10], [23], :

Type I - The predominance of pyramidal and extrapyramidal anomalies, in addition to ataxia and other signs, with early-onset and rapid progression.

Type II - With typical cerebellar ataxia, progressive external ophthalmoplegia and pyramidal signs that appear in middle age.

Type III - Late-onset and slow progression of peripheral signs, such as loss of proprioception and muscle atrophy.

Type IV - The rarest of all types, is characterized by the presence of Parkinson's signs, associated with the main clinical characteristics.

Within these four clinical sub-phenotypes defined here, type III is the most commonly observed among the several cases [24].

MJD pathology

Post-mortem pathological examinations of MJD brains showed, large atrophy of the cerebellum, oblong medulla, as well as cranial motor nuclei [25], [26]. Recently, it was shown that all pre-cerebellar nuclei and the thalamus are also affected [27], [28]. In addition, the gross weight of the brain was lower than that of individuals without a history of neurological or psychiatric illnesses [29]. Furthermore, MJD neuropathology includes severe neuronal loss in the anterior horn and Clarke's column of the spinal cord and in restricted brain regions such as dentate nucleus (cerebellum), pontine nuclei and locus coeruleus (brainstem), substantia nigra (basal ganglia) and other regions such as the thalamus, as well as subthalamic, red and cranial nerve nuclei [7]. Depigmentation of the substance nigra was also reported. It is also characterized by axonal neuropathy of peripheral motor and sensory axons [30].

The protein involved in MJD – ataxin-3 – is expressed in all cells and cellular compartments. When this protein is mutated, presenting an expanded polyglutamine tract, causes the disease and is prone to aggregate into Nuclear Intracellular Inclusions (NIIs). Despite the controversy around the relevance of these NIIs in the disease pathology, they constitute a hallmark of the disorder, being found in MJD patients brains [31], [32].

In order to better understand this disease, several mouse models of MJD have been created in recent years [33]. A summary of the main characteristics of these models can be observed in Table 1 and Table 2.

Table 1 – Summary of the MJD mouse models characterization at the pathological level. Adapted from [1].

General Information								
Neurodegeneration	**Cellular and subcellular transgene expression**	**Inclusions**	**(CAG(n**	**Promotor**	**Transgene**	**Age at onset (weeks**	**Genetic background**	
Purkinje cell degeneration and atrophic cerebellum	Purkinje cells	No description	79	L7 (Purkinje cells)	cDNA MJD1a Fragment with 79 CAGs; cDNA MJD1a full-length with 79 CAGs (no phenotype)	4 (ataxic phenotype)	no description	**Ikeda et al. 1997**
Peripheral neuropathy with demyelination and degeneration of the DRGs; cell loss in the DCN and Purkinje cells; astrocytosis	Ubiquous (cytoplasmic and nuclear)	Yes	64, 67, 72, 76 and 84	Human ataxin-3 (YAC)	All isoforms	75 (heterozygous), 6 (homozygous) (motor deficits)	C57BL/6J (mixed?)	**Cemal et al, 2002**
Substantia nigra: 38% decrease in TH-positive neurons Q71-C homozygotes	Ubiquous but stronger in brain and spinal cord	Yes (homozigosity)	71	Mouse prion protein	Isoform ataxin-3c	8 (tremor, hunchback, ataxic limbs)	B6C3F1/J (C57BL/6JxC3H/HeJ	**Goti et al, 2004**
Purkinje cell loss	Ubiquous but directed to brain and spinal cord (NES and NLS artificial signals)	Yes	70	Mouse prion protein	Isoform ataxin-3c	6 (tremos reduced activity, wide-based hindlimbs to stabilize the body in resting position)	C57BL/6N (mixed?)	**Bichelmeier et al, 2007**
Purkinje cells with shrunken cell body and less dendritic arborization	Ubiquous but directed to brain and spinal cord	Yes	79	Mouse prion protein	Isoform MJD1a HA-tag	20 (motor deficits)	FVB/N	**Chou et al, 2008**
Purkinje cells darkly stained	Brain (stronger in cerebellum but only in glia cells	Yes	77	Hamester prion (conditional expression)	Isoform ataxin-3c	8 (limb clasping)	C57BL/6 (mixed?)	**Boy J et al, 2009**
Purkinje cells darkly stained	Ubiquous (cytoplasmatic and nuclear)	Yes	148	Rat huntingtin (partial)	Isoform ataxin-3c	48 (reduced balance and coordination)	C57BL/6N	**Boy J et al, 2010**
Astrogliosis in substantia nigra and vestibular nuclei; scattered dark, shrunken cells in pontine and dentate nuclei and thalamus	Ubiquous (cytoplasmatic and nuclear)	No	94	CMV	Isoform ataxin-3c	16 /motor deficits)	C57BL/6J	**Silva-Fernandes et al, 2010**

Table 2 - Summary of the MJD mouse models characterization at the phenotypic level. Adapted from [1].

Phenotype parameters													
Ataxia	Gait disturbance	Weight loss	Hypoactivity	Motor uncoordination	Apperance	Limb clasping	Tremors	Lowered Pelvis	Limb strength deficit	Other	Phenotype		
Yes	Yes	X	No	X	Small	X	X	X	X	X	Ikeda et al, 1997	+/-	
X	Yes	Yes	Yes	X	Normal	Yes	Yes	Yes	Yes	X	Cemal et al, 2002	+/-	
X	Yes (++)	Yes (++)	Yes (++)	Yes	X	Yes (++)	Yes (++)	Yes (++)	Yes	Excessive grooming		+/+	
No phenotype	No phenotype	No phenotype	No phenotype	No phenotype	Normal	No	No	No	No	X	Goti et al, 2004	+/-	
Yes	Yes	Yes	Yes	Yes	Moribund (32weeks)	Yes	Yes	Yes	Yes	X		+/+	
Yes	Yes	X	Yes	X	Disheveled appearance (24 weeks)	X	Yes	X	X	X	Bichelmeier et al, 2007	+/-	
Yes	Yes	Yes	Yes	Yes	X	Yes	X	Yes	X	X	Chou et al, 2008	+/-	
Yes	Yes	Yes	X	Yes	X	Yes	X	X	X	Hyperactivity and spastic head movements (homozigoty)	Boy J et al, 2009	+/+	
X	X	X	X	Yes	X	X	X	X	X	Hyperactivity	Boy J et al, 2010	+/-	
No	No	No	Yes	Yes	Normal	No	No	No	No	X	Silva-Fernandes et al, 2010	+/-	

Genetics

Since MJD is an autosomal dominant inherited disease, this means that the presence of the mutation in a single allele is capable of giving rise to the disease. This implies that when an individual with MJD has children, there is a 50% chance for those children to have the same disease. The disease is caused by an expansion of a CAG repeat tract within the ataxin-3 gene (ATXN3) which above a certain threshold is considered pathogenic. The CAG repeat is highly unstable, but usually CAG repeats higher than 45 causes disease. Interestingly, above the pathogenic threshold the CAG tracts are also unstable across generations, and they may expand or contract, biases towards expansion or contraction depending on the sex of the transmitter parent: while maternal meioses are generally more stable or tend to lead to contractions, paternal transmissions display more instability and tend to lead to expansions. This intergenerational instability leads to a phenomenon called anticipation, since the disease symptoms appear earlier and are more severe in later generations [11], [34], [35].

A few years after the disease was first described, the disease-causing gene was identified and mapped on chromosome 14.q32.1 [36], [37]. A year later, the MJD1 gene (later called ATXN3) was cloned, and the authors noted that the mutation (expansion) of the CAG tract was found only in the affected people [38]. All this knowledge acquired, enabled the creation of a molecular diagnosis for MJD, based on the length of the CAG repeats and, consequently, allowing the confirmation of the disease in families with different origins [35], [39].

In 2001, the genomic structure of the ATXN3 gene was discovered, which led to the knowledge of the number and size of exons and introns. The approximately 48Kbp gene, containing 11 exons and the CAG tract present in exon 10, has the ability to encode at least four different transcripts with variable sizes: 1.4, 1.8, 4.5 and 7.5 Kb, possibly through differential splicing and polyadenylation signals [40], [41]. Today, 56 more splice variants of the ATXN3 gene are known, with some that are only seen in affected individuals [42], [43]. Regarding the biological importance of these variants, there is still no sustained information. In the brain, ATXN3 is expressed in neurons, although, at lower levels, they are also present in glial cells. It is important to note that despite the ubiquitous expression of ATXN3, only some neurons are affected and die; this selectivity is not yet understood.

Different mouse models for the same disease

Nowadays, there is still no treatment available to cure or delay the onset of MJD, therefore a high need for further studies in live models is of extreme importance. Several models of mice have been created to facilitate the understanding of the disease and the development of a therapeutic strategy. Rodent models, especially mice, are the species most used in research, mainly by their smaller size (easy to maintain) and the ease of genetic manipulation presented. It is possible to generate relatively large numbers of mice, kept in controlled laboratory environment. Being mammals, their genomic, anatomical, and physiological similarities to humans are of extreme importance [44]. The use of patient's brain tissues has technical problems, such as autolysis caused by long postmortem delays. Those can be overcome using mouse models.

One of the transgenic mouse models of MJD was generated in the Lab of Professor Maciel, at the Life and Health Science Research Institute (ICVS), Universidade do Minho, Braga, using the human cDNA (isoform1, clone MJD1-1), carrying the repeated tract encoding for 135 polyglutamines, under the control of the CMV promoter (cytomegalovirus), a strong and ubiquitous promoter [45].

This transgenic mouse model – the CMVMJD135 (Figure 1) - shows some important characteristics that mimic MJD, including motor-related symptoms that appear gradually and progress during life. In fact, this model fits well in the three main criteria used to validate a mouse model: (i) construct validity, stating that the animal model has to carry the same biological features that cause the human disease, such as the same genetic alteration or anatomical abnormality, (ii) face validity, that comprises a conceptual correspondence to the behavioral phenotype (symptoms) and endophenotype (physiological/anatomical alterations) of the human disease, and (iii) predictive validity, where the response to treatments (preventive or curative) should be analogous between mice and humans [45], [46].

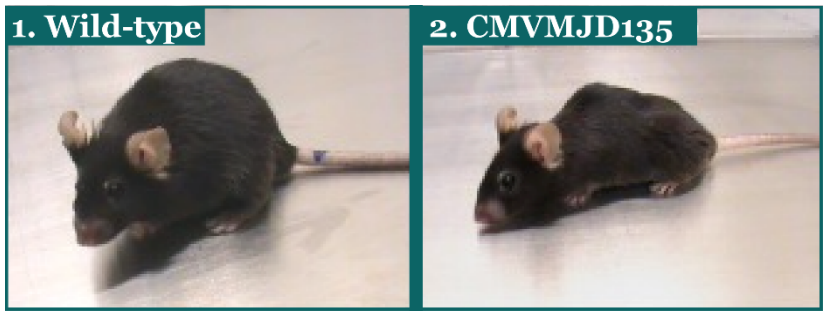

Figure 1 – The transgenic mouse model of MJD: (1.) Wild-type mouse; (2.) CMVMJD135 mouse at 40 weeks of age. Adapted from [45].

Some of the main characteristics of the CMVMJD135 mouse model are [45]:

- Progressive Neurological Deficits.

- Presents a visibly abnormal gait, an unhealthy appearance, an abnormal body posture, limb clasping and limb tonus deficit, decline in body weight, dragging of the feet, uniformity of step alternation, a significant decrease in the stride length, swim significantly slower (in addition, abnormalities in their swimming movements, adopting a twisted posture, and kicked in an uncoordinated), significant differences in performance in the hanging wire grip test, loss of hindlimb tonus resistance, a decrease in forelimb strength and hindlimb clasping, tremors and the decreased locomotor and exploratory activity.

- Reduced Brain Weight (~5 %), reduced volume and total cell number in pontine nuclei indicating neuronal demise in this region and presence of ataxin-3 inclusions in the nucleus of neuronal cells in different regions of the CNS including the pontine nuclei, reticulotegmental nucleus of the pons, spinal cord neurons, the facial nuclei, anterior olfactory nuclei, ventral tenia tecta, inferior olive, the dentate nuclei, locus coeruleus and the cuneate nuclei.

Overall, this model is a powerful tool to search for therapeutic strategies for MJD/SCA3, being capable of mimicking many aspects and peculiarities of the disease and can also be useful to dissect the initial cellular and molecular events in the pathogenesis of this disorder.

Therapeutic strategies for MJD/SCA3

Over all these years several researchers have sought a therapeutic strategy for MJD. According to a recent review, (Costa, M. do C. (2020), some strategies, such as DNA-Targeted Treatments, RNA-Targeted Strategies, approaches Targeting protein quality

control Pathways, Strategies Targeting Cellular homeostasis, show that hold promise as future effective treatments for this incurable disease. However, no disease-modifying treatment is yet available, and the recommendations are mostly derived from other neurodegenerative diseases [47].

Still, there are some symptomatic treatments available such as genetic counselling, speech evaluation and swallowing physiotherapy programs. Symptomatic treatment for cramps, pain, sleep disorders, depression, dystonia, and parkinsonism are important to provide a better quality of life for patients, slowing the onset of symptoms and mitigating the impact of symptoms throughout the life of patients [1].

Looking at the case of Parkinson's disease (PD), with aging, there is a functional decline and the physical activity levels tend to decrease [48]. Physical exercise improves physical fitness, which reveals benefits in terms of neuroplasticity and the ability of the brain to repair itself [49]. In the case of PD patients, they reduce physical activity levels faster, showing strength and functional capacity levels lower than healthy patients, [50]. However, the loss of muscle strength is not simply consequence of inactivity and of ageing, but a primary symptom of PD [51].

Animal models have proved that physical exercise can be a therapeutic strategy in slowing down PD symptoms, allowing the better functioning of the body, a better quality of life related to health, strength, balance, and gait [52]. This result could be related to the possible release of neurotrophic factors and to increase brain oxygenation, which together promotes cell growth and survival [53], [54].

Contrary to what has already been done in PD, it has not been described that voluntary physical exercise may be beneficial for patients with MJD. That said, it is a therapeutic strategy to study with good indications of other diseases with similar symptoms.

Evolution of home cages for laboratory mice

In the laboratory environment, it is known that mice spend 99% of their time in the home cage, and that they are naturally more active at night. However, most of the experiments take place during the day. In the case of behavioral tests, only data from that specific moment is collected, and these are performed outside the home cage and end up inducing acute stress due to the handling of the animal. It is also known that there are several environmental factors that cannot be controlled in the animal house, such as changes of bedding, the presence of people in the room and the resulting noise, etc [55]–[59]. Among others, in an era of technological evolution, these are some

aspects that need to be addressed and overcame. The automatic monitoring of home cages has emerged as a way to accomplish this and has been progressing enormously within the development of Individual Ventilated Cages (IVCs) systems. Such a technologically advanced system allows to collect spontaneous animal activity data without any human intervention. As benefits, this animal monitoring system not only has a potential impact on extracting relevant information from a scientific perspective, but also offers improved animal welfare [60], [61], [62]. The observation of home cages is, thus possible 24 hours a day, 7 days a week, allowing the collection of data related to the activity and behavior of the animals, such as tracking the animal inside the cage, during the entire period of accommodation in the rack, which previously would have been unknown due to the inability of continuous observation [63], [64], [65]. Therefore, this capability provides a completely new set of information for researchers performing animal experimentation [66].

Currently, there are numerous systems and technologies capable of monitoring home cages[62], from video cameras [65], [67], beam braking systems [68], and force transducers [69] to other techniques based on passive infrared, piezoelectric and microwave [70]–[72]. Each technology always has its advantages and disadvantages. In these examples given above, there is a limitation of scalability of the system. When the number of cages is high the data generated, the computational power and mechanical configuration are a major disadvantage. In addition, they may require dedicated staff to manage the various interventions required [73].

Digital Ventilated cages - DVC®

The Digital Ventilated Cage (DVC®, Tecniplast, Buguggiate, Italy) (Figure 2) is a commercial system capable of collecting various information from animals living in an IVC system, without disturbing them and while their cage is placed in the rack. Detailed technical information regarding this commercial system can be found in the attachment “support information about the DVC®”, as provided by Tecniplast.

After the installation and configuration of the DVC®, human intervention is no longer necessary for data collection, as the system continuously monitors and stores all the data for each cage position, 24 hours a day, 7 days a week, automatically. It is possible to check in a data history, a whole list of events that occurred in any of the cages [74].

In addition to this, the DVC® is equipped with a series of features capable of helping the management of daily tasks of by the vivarium technicians, contributing for a more efficient work, and helping to keep animals in the best conditions and therefore in good

welfare state. Within these functionalities, one should highlight the analysis of the bed conditions: the humidity level of the bed is analysed, and the system is thus able to suggest a better bed changing schedule instead of following a fixed weekly (or biweekly) approach. Information regarding the availability of water and food, cage flood detention, inactivity inside the cage or strange activity level in a cage, are indicators of welfare that are all automatically registered by the system [74]

Another advantage of the DVC® is to provide automatic information regarding the location of all (registered) cages allowing an accurate automatic census and helping managers with the per diem calculations [74].

With all its digital technology, the DVC® turns out to be a real digital vivarium, bringing standardization in housing, animal welfare and, at the end, better management of the vivarium.

Figure 2 – Digital Ventilated Cages (DVC®), a home-cage rack monitoring system that allows the continuous detection of spontaneous animal activity 24h/7 days a week.

Spontaneous animal locomotion

The DVC Plate® under each cage in the DVC®, consists of 12 electrodes connected to an integrated circuit that measures electrical capacitance continuously. Since the capacitance varies depending on the presence or absence of matter in the area covered by each electrode, its measurements are affected, for example, by the presence of water or animals. Of notice, the materials with high water content are described by high values of the relative permittivity (in relation to air), which in turn are related to a higher capacitance [63]. As the mice have a high-water content, their movements induce significant changes in the capacitance measured by the electrodes and, therefore, it is possible to track these changes over time as an indicator of the animal's activity. Additionally, if there is no change in the material above the electrode, the capacitance remains substantially unchanged [63]. The functioning of the DVC can be appreciated through the example in (Figure 3).

All DVC® Plates installed in a DVC® rack or group of racks, are then connected to a specific computer, known as the DVC® Master, which provides power and data connection. The DVC® Master plays a key role in collecting raw data from each of the DVC Plate®, with the option of both transferring the collected information to a web-based software application or to a dedicated storage device for processing the entire information collected [74].

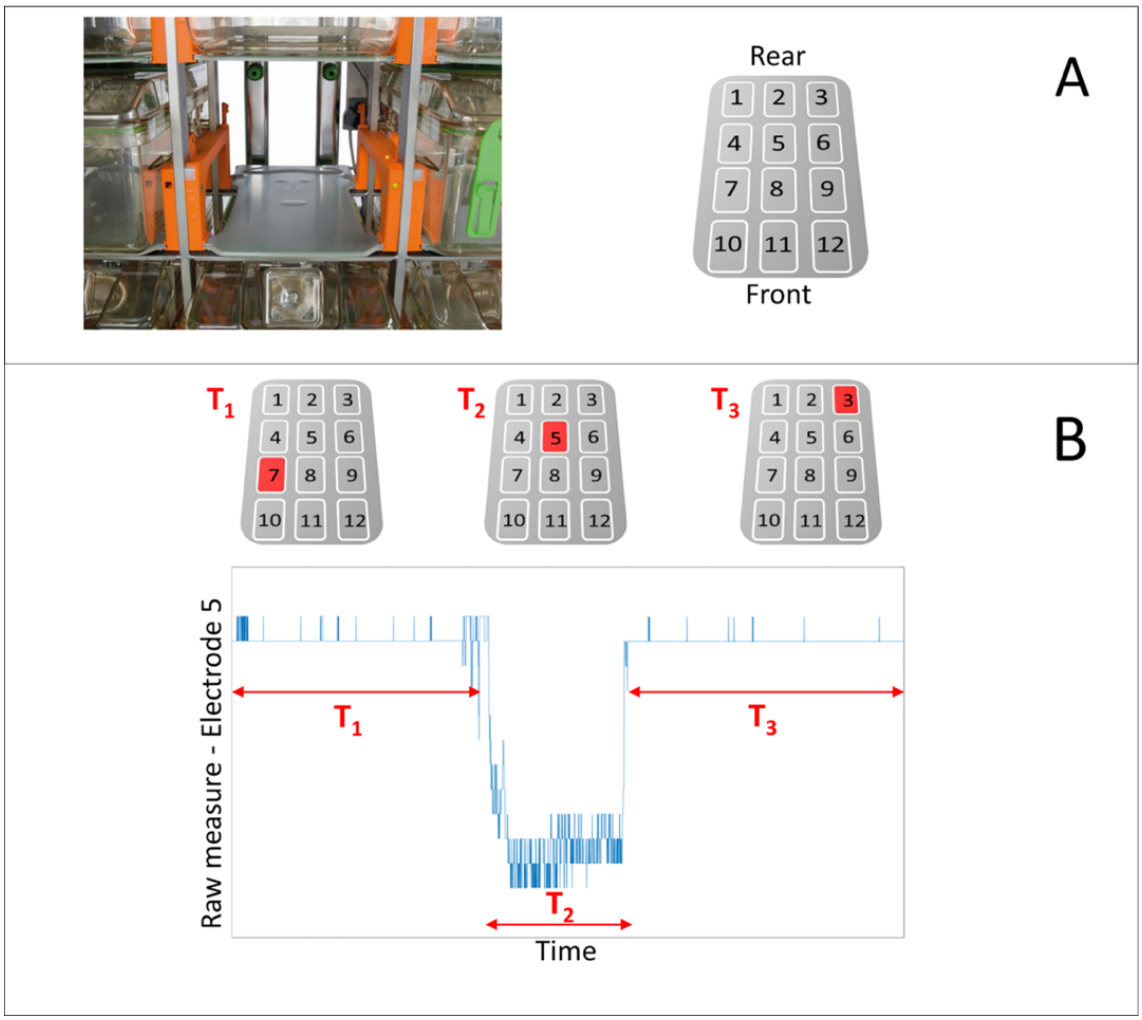

Figure 3 - DVC® system description. A) Standard IVC cage position equipped with DVC® sensing technologies. The grey board contains the 12 electrodes. Electrodes numbering is shown in the right panel; B) shows example of raw signal measured from electrode 5 when a mouse stays for T1 seconds in a cage area close to electrode 7, then moves towards and across electrode 5 during T2 seconds and then moves on to electrode 3 staying there for T3 seconds. Adapted from [66].

Running Wheels in the DVC® as a therapeutic strategy for neurodegenerative disease and as an environment enrichment

Running wheels (RW) can be used as a behavioral readout and their effects in terms of physical exercise have been widely used to assess neurobiological processes, such as brain plasticity, neurogenesis, sleep-wakefulness, and ageing [75]–[81]. Some studies suggest that running wheels might improve welfare when provided to laboratory animals as an extended opportunity for locomotor activity [82]–[84].

These DVC® Running Wheels (Figure 4) were specifically developed for use in the DVC®, capable of providing data related to distance travelled, speed and number of revolutions. They allow for clockwise and counterclockwise rotation so that there is no interference with the mouse spontaneous use of the RW [85]. In this way, the RW allows the practice of voluntary physical exercise as a form of positive physical enrichment for the animal inside its home cage.

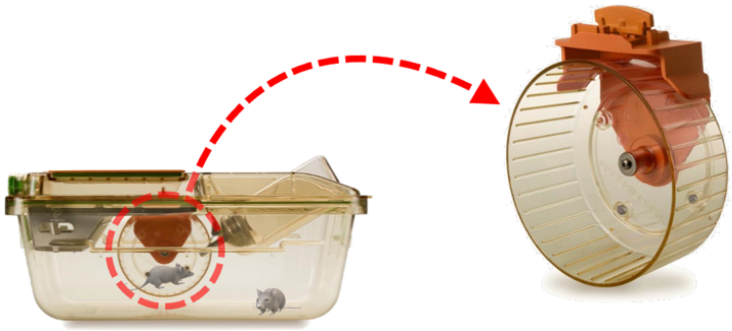

Figure 4 - The DVC® Running Wheel allows to automatically record data on the use of the RW by mice. This automatic data collection will provide not only information on the use of the RW but also the total distance covered.

Present study hypothesis

In this study, two main hypotheses were postulated:

- (i) The DVC® automated system is able to register and detect the motor phenotype of the CMVMJD135 animals without the experimenter intervention.

- (ii) The RW adapted for the DVC® system has a therapeutic effect in the CMVMJD135 animals: physical exercise is known to improve several phenotypic aspects in different neurodegenerative diseases; thus, we hypothesized that voluntary exercise could improve the motor-related symptoms of MJD mice.

- To evaluate hypothesis (ii) we have used the RW, which was available in the cage so that the mice could run voluntarily. We had 4 different groups: Wild-Type (WT) and Transgenic groups (TG) without access to the RW, and WT and TG with access to the RW (WTRW/TGRW).

Chapter 2 – Objectives

The main goal of this dissertation was to understand the influence of voluntary physical exercise as a potential therapy to improve motor dysfunction in the mouse model CMVMJD135 of MJD.

To achieve this, the study was divided into two main goals:

i. **To understand if the DVC® is able to detect the motor phenotype of the CMVMJD135 Animals:** by assessing the activity patterns of the different groups, over time, without the intervention of the experimenter.
ii. **To evaluate if voluntary exercise improves the motor-related symptoms of MJD mice by:**

    - Determining the distance covered by the mice in the running wheel which is available in the cage;

    - Performing additional behavioral tests, which are usually the standard for characterizing the disease establishment and progression in MJD mouse models, to understand the influence of spontaneous activity within the cage and the performance in the RW by each group.

In this way, we aimed to evaluate:

- The activity pattern of TG mice when compared to the WT animals along disease progression, and test if the DVC® could give us reliable indicators of animal welfare for such disease models;

- The impact of the presence of RW in MJD progression.

Complimentarily, we have used additional behavioral tests, standardly used for characterizing the disease establishment and progression in mouse models of MJD, along with evaluating the spontaneous movement within the cage and the distance travelled on the RW, as provided by the DVC®.

Chapter 3 – Materials and Methods

Animals and housing conditions

Female CMVMJD135 mice (background C57BL/6J) were used, and they were sequentially distributed among the 4 groups (WT; TG; WTRW; TGRW), 2 animals per cage, housed under the following conditions: (GM500 cage (Tecniplast, Buguggiate, Italy); in which the animals assigned to the RW groups have Running Wheel (Tecniplast, Buguggiate, Italy).

All animals were kept under the following laboratory conditions: artificial light cycle 12h/dark (lights on from 8am to 20pm), with ambient temperature of 21 +-1°C and a relative Humidity of 50-60%; with corn Cob bedding (Scobis Due, Mucedola SRL, Settimo Milanese, Italy) and soft paper as environmental enrichment standardized in the animal facilities (Renova Active Maxi, Torres Novas, Portugal); the mice received a standard diet (4RF21, Mucedola SRL) and water ad libitum.

Ethics statement

This project and all animal protocols were approved by the national authority for animal experimentation (DGAV020317, 28-09-2016) and by the Animal Ethics Committee of the Life and Health Sciences Research Institute, University of Minho (Braga, Portugal).

The experiments were designed under the principles of refinement, reduction, and replacement. Health monitoring was performed according to (Federation of European Laboratory Animal Science Associations) FELASA guidelines, where the Specified Pathogen Free health status was confirmed by sentinel mice maintained in the same animal housing room. To minimize discomfort, stress and pain to the animals, humane endpoints were defined and included a 20% reduction of the body weight, incapacity to reach food and/or water, presence of wounds in the body or signs of dehydration. Animals were handled from beginning of the study by the same experimenter who performed all behavioral tests and changed their cages.

All procedures were conducted in accordance with European regulations (European Union Directive 86/609/EEC). Animal facilities and the people directly involved in animal experiments are certified by the Portuguese regulatory entity — Direção Geral de Alimentação e Veterinária (DGAV).

Genotyping

Animal identification and tail biopsy

At weaning, at 3 weeks of age, the animals were identified with an ear punch (using an ear clip) and a tail biopsy (2-3mm) was collected for further DNA isolation and genotyping procedures (MJD genotyping protocol).

DNA extraction

In this study, it was used one chemical method, the Citogene (Citomed) DNA isolation kit, to isolate DNA following the manufactures instructions Briefly, to disrupt the cell membranes and release the DNA along with the broken proteins 300 µl of Cell Lysis solution and 2 µl of Proteinase K (Novagene) were added to the tail tissue, followed by incubation overnight at 55oC (Binder, Inc.). After centrifugation at 13000 rpm, for 8 minutes, the supernatant (containing the DNA) was transferred to a new tube containing 300 µl of 100% isopropanol for precipitation, followed by centrifugation at 13000 rpm for 8 minutes. After removing the supernatant, 300 µl of 70% ethanol were added to the pellet and the samples were centrifuged at 13000 rpm for 5 minutes. The supernatant was discarded and 30 µl of DNA Hydration solution was added to the DNA pellet. The isolated DNA was stored at 4°C and used for the MJD genotyping protocol.

Genotyping protocols

For the genotyping of the mice, a protocol established and described by P. Maciel Lab [86], was used to determine the mouse genotype and to determine the length of the CAG repetition of transgenic animals.

The primers mmMJD89 (5′- CAAAGTAGGCTTCTCGTCTCCT- 3′) and mmMJD54 (5′-AGTGCTGAGAACACTCCAAG-3′) were used to amplify the mouse endogenous Atxn3 gene (546bp), which was used as an internal control for the PCR. The primers TR1((5′-GAAGACACCGGGACCGATCCAG3′) and TR2(5′-CCAGAAGGCTGCTGTAAAAACGTGC-3′) were used to amplify the transgene (454bp). Briefly, for the identification of WT/transgenic animals, 12 µl of the reaction mixture and 0.5 µl of each DNA were added to respectively PCR tube. The reaction mixture was composed by nuclease free-water, the primers mentioned above (mmMJD89 (16ng/uL), mmMJD24 (16ng/uL), TR1(10ng/uL) and TR2 (10ng/uL)) and Master mix Supreme from NZYTech [87]. PCR cycling conditions were as follows: 95°C for 5 minutes followed by 35 cycles of denaturing at 95°C for 1 minute, annealing at 64°C for 1 minute, extension at 72°C for 1 minute and a final extension at 72°C for 5 minutes.

Then, a solution with 1,5% agarose powder and Tris Acetate-EDTA 1x buffer (TAE, used as a running buffer in the electrophoresis process) was heated in a microwavable flask until the agarose was completely dissolved in the TAE buffer. Greensafe Premium fluorescent dye (NZYTech) was added for nucleic acid visualization. These dyes bind to the DNA and afterwards allow the visualization of the DNA under ultraviolet (UV) light. The solution was placed into a gel tray with the well(s) comb(s) in place until the gel completely solidified. Next, the agarose gel was transfer to the electrophoresis unit and TAE 1x were added to the unit until covered the gel. The DNA molecular weight ladder Gene Ruler TM 1kb (ThermoFisher Scientific) was loaded into the first lane of the gel, while each sample was loaded very carefully in the other wells. Gels were visualized with GelDoc Go Imaging (Biorad), using the ImageLab software. An example of the different bands can be seen in Figure 5.

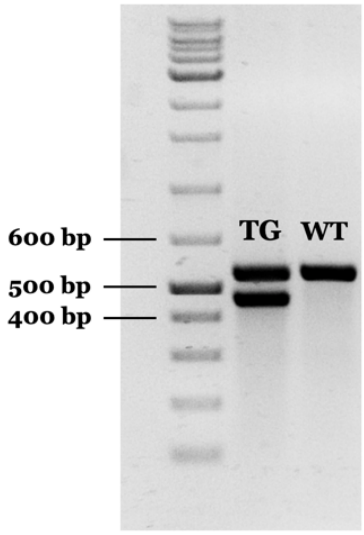

Figure 5 - Example of an agarose gel containing PCR samples from a transgenic (2 bands) vs. Wild-Type (1 band) animal, respectively from left to right.

For the determination of the CAG repeat, the PCR products are analyzed by GeneScan to determine the CAG repeat length, as an outsourced service as described previously [86].

Behavioral analysis

The behavioral tests were performed during the daytime period and the animals were tested at 30, 33, 36 and 39 weeks of age (Figure 6).

Experimental design

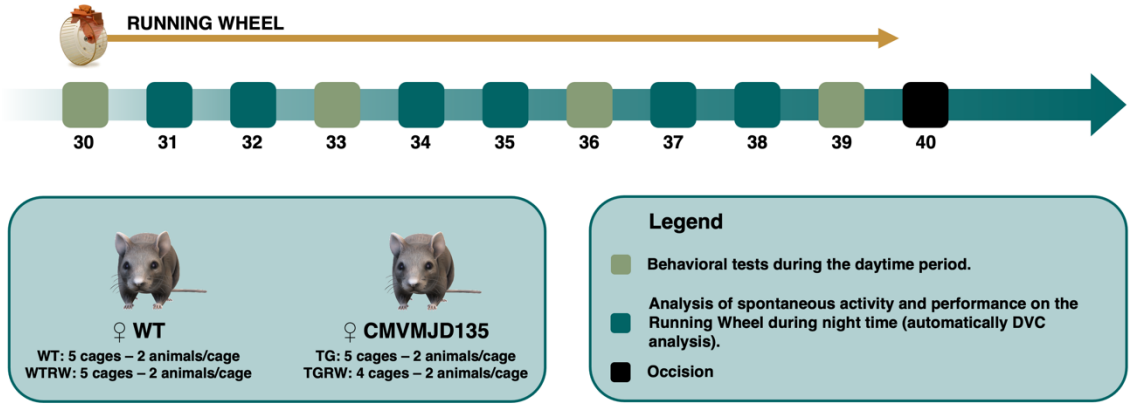

Figure 6 – Timeline of the behavioral tests. The scheme illustrates the order by which all behavioural tests were performed, as well as the number of cages per group.

Body Weight

All mice were weighed at each time point using an appropriate dynamic range scale (OHAUS, mod. EX2202/E) to measure the bodyweight of the mice as they move.

Beam walk balance test

This test was used to assess balance and fine motor coordination. The beams consisted of long strips of PVC (1 m) with square or round cross-sections of different thicknesses. The beams are placed horizontally, 50 cm above the countertop surface (protected with soft sponges to protect the animals in case of falls), with one end mounted on narrow support and the other end attached to a closed dark box (20 cm square) where the mouse can escape and feel safe (Figure 7).

The animals were trained for 3 days on the square beam (12 mm). On the fourth day, they were tested on the training beam (12mm) and on a round beam (11mm). Due to the increasing in the difficulty of the animals to perform the task, at 33 weeks of age, the round beam (11 mm) was replaced by another thicker round beam (28 mm). If the animal falls or turns on the beam, it will be considered a failed trial. Each animal will have the opportunity to fail twice on each beam. The time it takes the animal to cross the beams will be registered and will be discounted if the animal stops in the beam [86], [88].

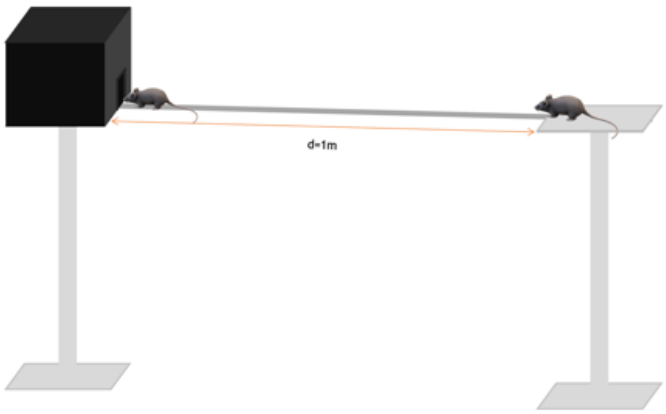

Figure 7 – Representative image of the beam walk test.

Motor swimming test

To analyse voluntary locomotion, mice will be trained for 2 consecutive days (3 trials per animal) to traverse a clear Perspex water tank to a safe platform at the end. The Perspex tank is 100 cm long and the platform at the end is made from black Perspex. The latency to cross the water tank will be measured from a distance of 60 cm (the tank is labelled with a blue line to mark the initiation) (Figure 8). Water temperature will be monitored to 23ºC using a thermostat. Mice will be tested for 3 consecutive days (2 trials per animal) and latency to traverse the tank registered by the experimenter with a chronometer [86], [88].

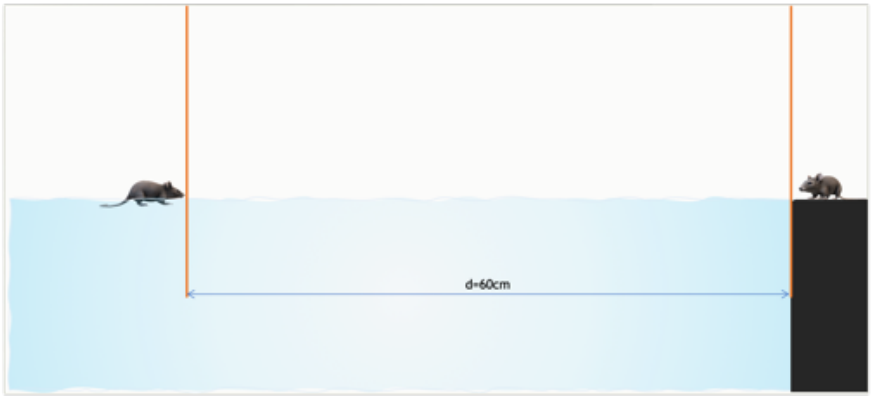

Figure 8 – Representative image of the motor swimming test.

Horizontal spontaneous activity

The mouse will be transferred to the center of a 15-labelled arena (55 × 33 × 18 cm) (Figure 9) and the experimenter will count the number of squares travelled by the animal in the arena for 1 minute [86].

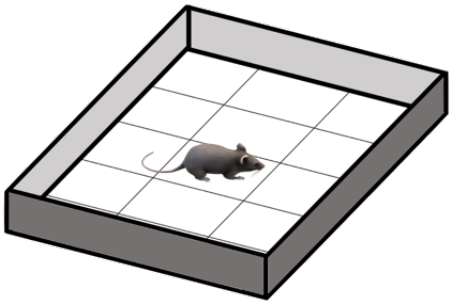

Figure 9 – Representative image of the horizontal open arena.

Hanging wire grid

The mouse will be placed on top of a metallic grid and inverted 180º towards the surface of the bench (Figure 10). The latency to fall from the metallic grid will be recorded by the experimenter. The maximum time allowed for the test will be 120 seconds.

In case the mouse is able to climb to the top of the metallic grid, the experimenter will consider that the mouse can withstand the 120 seconds inverted [86].

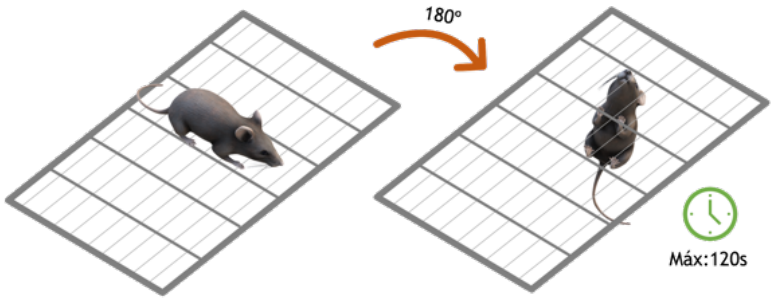

Figure 10 – Representative image of the hanging wire grid test.

Strength to grab

The animals will be pulled by the experimenter from the tail on a metallic grid, allowing the mice to exert strength against the strength applied by the experimenter (Figure 11). The strength will be recorded using standardized scores (0 - Normal; 1 - Abnormal) [86].

Figure 11 – Representative image of the strength to grab test.

Hindlimb tonus

The mouse will be properly restrained, and the experimenter will softly push the hindlimb towards the body of the animal (Figure 12). The applied force by the mouse against the experimenter finger will be scored (0 = normal resistance; 1 = mild resistance; 2 = no resistance) [86].

Figure 12 – Representative image of the hindlimb tonus test.

Clasping

The mouse will be lifted by the experimenter from the tail at a certain height and will then bring the mouse to a metal grid assessing whether the mouse retracts any of the hindlimbs (Figure 13). The experimenter will assign a standardized score (0 - no clasping; 1 - mild: present in one paw; 2 - present in two paws) [86].

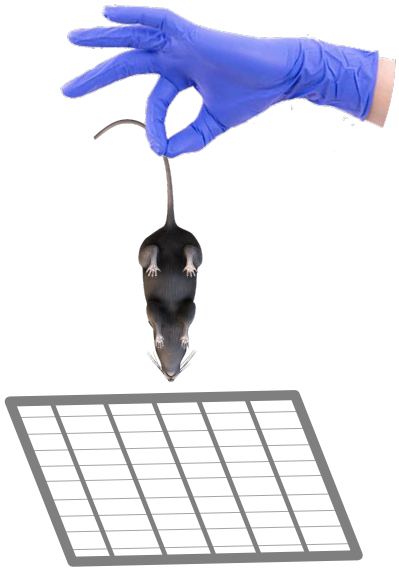

Figure 13 – Representative image of the clasping test.

Footprinting analysis

The footprint pattern will be performed to assess gait. To obtain footprints, the hind and forepaws of mice will be painted with non-toxic black and red ink, respectively. A clean rectangular sheet of paper will be placed on the floor of the runway for each run. The animals will then be allowed to walk along a corridor of 100 cm long x 4.2 cm wide x 20 cm height, in the direction of an enclosed black box. An inclined corridor will be used instead of a horizontal corridor, since the mice tend to run upwards to escape (Figure 14). Each animal will be allowed to achieve one valid trial per each time point analysed. To evaluate the severity of foot dragging through age, the footprinting pattern will be classified at each time point considering six consecutive steps (0 = absent; 1 = mild, up to three steps; 2 = severe, more than three steps out of six) [86].

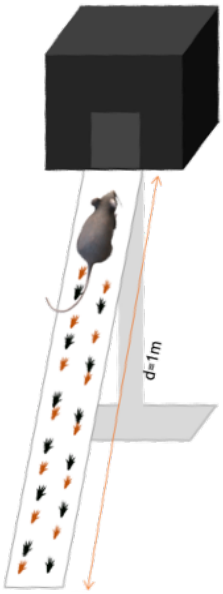

Figure 14 – Representative image of the footprinting test.

Voluntary exercise analysis

Spontaneous locomotion

Digital ventilated cage (DVC®) collects information from the DVC® board (Figure 15). The DVC® board is composed of 12 electrodes connected to an integrated circuit that continuously measures their electrical capacitance. Thus, movements across the electrode array are detected and recorded as alterations in capacitance. By applying custom-designed algorithms to the collected data it is possible to infer information regarding in-cage animal activity [63], [66].

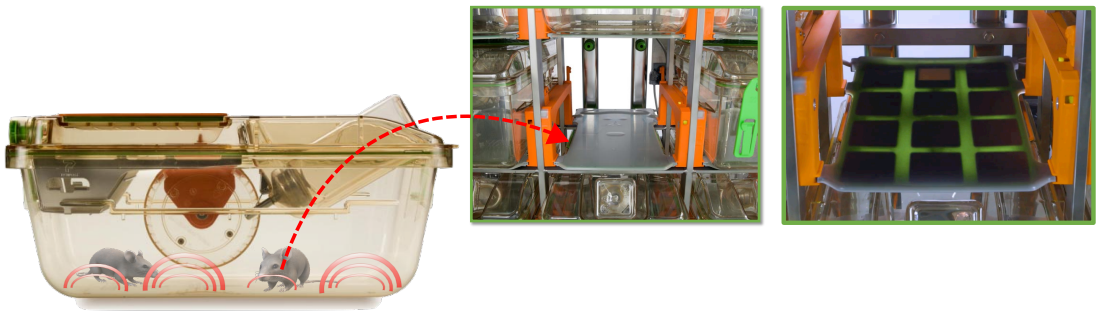

Figure 15 – Representative image of the home-cage rack and the DVC® board. Adapted from [74], [85].

DVC® Running Wheel

The DVC® Running Wheel (Figure 16) allows to automatically record data on the use of the Running Wheel by mice. This automatic data collection will provide not only information on the use of the Running Wheel but also the total distance covered [85].

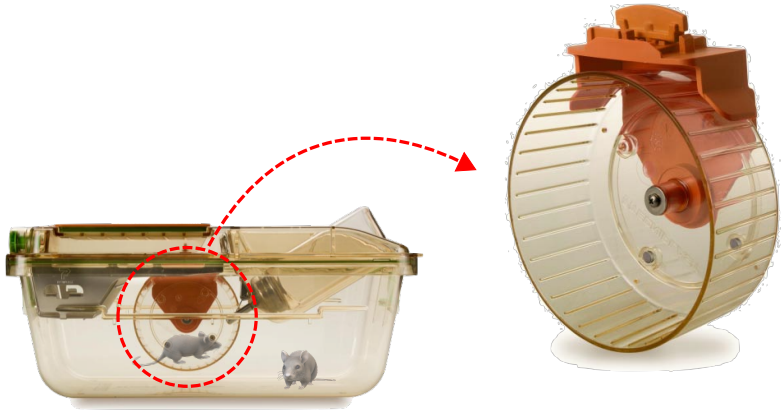

Figure 16 – Representative image of the Running Wheel. Adapted from [85].

Statistical analysis

The G*Power 3.1.9.2 software was used to calculate the sample size, based on a power of 0.8 (obtained from previous data) [89] and a significance level of 0.05, for all statistical tests. The final number of animals was 48 animals, but due to some initial problems in the study, only 38 animals were used.

Statistical analysis was performed using IBM SPSS statistics 22.0 (SPSS Inc., Chicago, IL).

Behavioural data were analysed by a repeated-measures ANOVA when variables were continuous or presented a normal distribution. The assumption of normality was assessed by qualitative analysis of Q-Q plots and frequency distributions (z-score of skewness and kurtosis) as well as by the Kolmogorov-Smirnov and Shapiro-Wilk tests. The assumption of homogeneity of variances was evaluated by Levene's test.

Regarding Repeated Measurements, to the comparison of means between 4 groups, the one-way analysis of variance (ANOVA) was used, followed by Tukey HSD or Dunnett T3's test (when data passed on the assumption of homogeneity of variances or when the populations variances were not equal, respectively).

Regarding non-normally distributed data and/or for the comparison of medians of discrete variables across time-points, Independent-samples was carried out, with pairwise comparisons through the Kruskal-Wallis statistic test.

Continuous variables with normal distributions (Kolmogorov-Smirnov test $p > 0.05$) were analysed with the Student t-test or two-way ANOVA (Factors: genotype and treatment). Behavioral data were subjected to the non-parametric Mann-Whitney U-test when variables were non-continuous or when a continuous variable did not present a normal distribution (Kolmogorov-Smirnov test p 0.05).

All data are presented as means ± S.E.M. In all cases, statistical significance was set at $p \leqslant 0.05$ (two-tailed).

GraphPad Prism 8 was used to create Figures. All statistical information is reported in the supplementary data.

Chapter 4 – Results

To our knowledge, there are no studies on MJD mouse models where an automated system was used to evaluate motor-related phenotype 24/7. Here, our goal was to assess the ability of a fully-automatized and experimenter-independent rack system to detect the progressive symptoms of the CMVMJD135 mouse model of MJD and to evaluate voluntary physical exercise as a potential therapy for the disorder, by adding running wheels to the home cage of the mice. Along with the automated phenotype detection, a standardized battery of motor tests already validated for CMVMJD135 mouse was performed.

First, and because the CAG repeat length was not known when the transgenic animals were randomly assigned to the different experimental groups, it was possible to observe that the CAG repeat was not significantly different between groups (Figure 17a). Nevertheless, we have noticed that the distribution of the CAG repeats where not homogenous between groups which led us to analyse the swimming performance of the individual animals accordingly to their CAG repeat. In fact, no differences were found between swimming performance among the different CAG repeats (Figure 17b). This data allowed to safely conclude all the other parameters analysed without any bias.

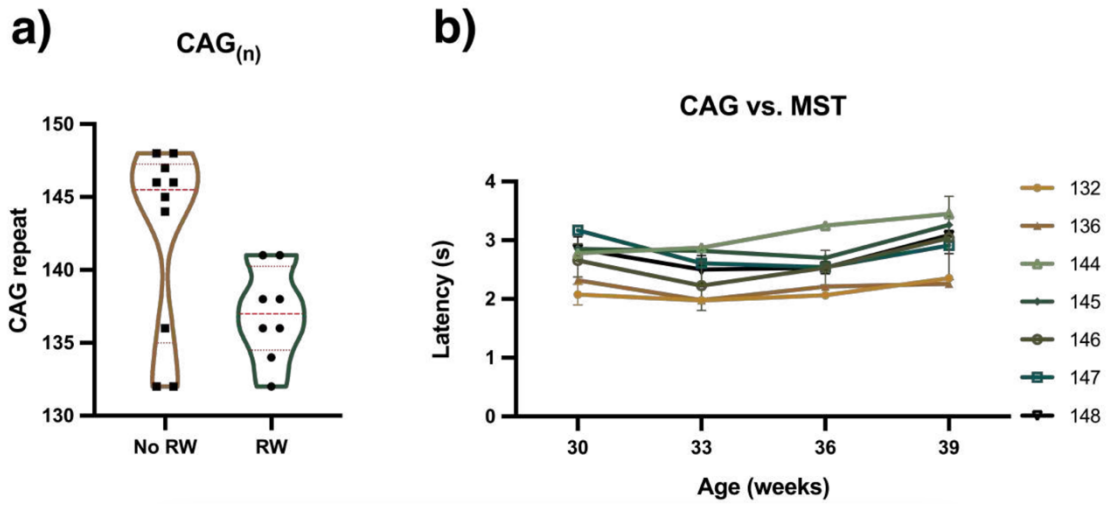

Figure 17 - CAG repeat length among the experimental groups – with and without running wheel and its influence on swimming performance. (a) The CAG repeat mean in the animals without RW was 142.40 while in the RW group was 137.00. Mann-Whitney U t-test (n=8-10 animals/group). **(b)** Swimming performance on the motor swimming test. No differences were found between CAG repeat length and animals' performance along time. Repeated-measures ANOVA; n=8-10/group.

Voluntary physical exercise on fully-symptomatic mice had mild effects on swimming performance

The swimming ability, measured by the latency to traverse a water tank, was evaluated in fully-symptomatic animals, starting at 30 weeks of age. TG mice showed a significantly worst performance over time when compared to their WT-littermates ((F(3,32) = 31,998, p<0,001, ω2=0,750). Surprisingly, the WTRW mice showed a slightly worse phenotype when compared to WT animals in specific timepoints (see Table 3 and Figure 18). Additionally, and although not statistical different, TGRW animals showed a better performance in their swimming ability when compared to TG mice without a RW.

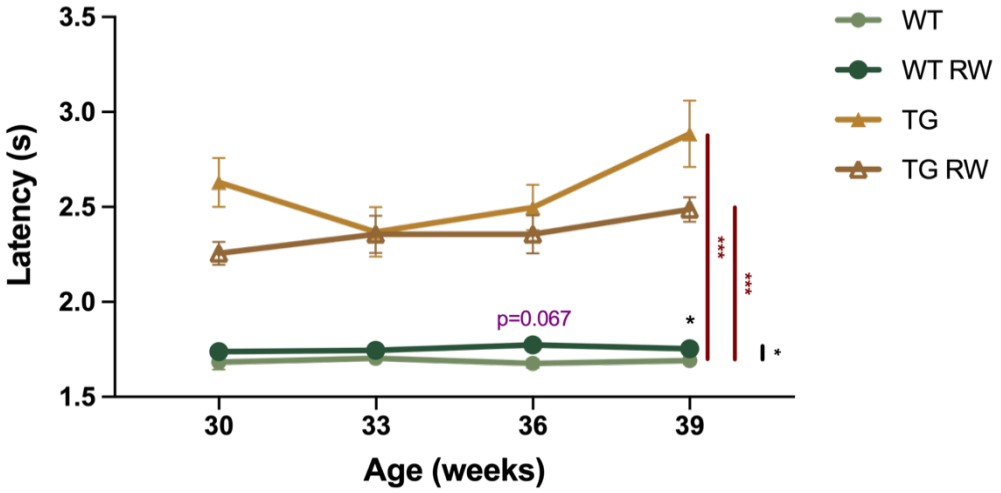

Figure 18 - Swimming performance of the CMVMJD135 mouse model. In the motor swimming test, the mice latency to reach the safe platform was measured during three consecutive days. Analysis of the total mean revealed significant differences between WT and TG animals (n= 8-10/group), but no effect of the RW was observed. Repeated Measures ANOVA and One-way ANOVA using Dunnett T3 Post-Hoc analysis. Symbols represent the mean ± SEM of the different groups; *, *** represent the p⩽ 0.05 and 0.001 respectively.

Table 3 – Results of statistical analysis of the motor swimming test: multiple comparisons between groups. One-Way ANOVA; *, **, *** represent the p⩽ 0.05; 0.001.

Samples	Weeks				
	30-39	30	33	36	39
WT vs. TG	0,000 ***	0,000 ***	0,040 *	0,000 ***	0,000 ***
WT vs. TGRw	0,000 ***	0,000 ***	0,001 **	0,001 **	0,000 ***
WT vs. WTRw	0,028 *	0,774	0,973	0,067	0,022 *
WTRw vs. TG	0,001 **	0,000 ***	0,005 **	0,001 **	0,001 **
WTRw vs. TGRw	0,000 ***	0,000 ***	0,002 **	0,003 **	0,000 ***
TGRw vs. TG	0,572	0,109	1,000	0,924	0,258

Animals with access to RW showed higher body weigh

Body weight was registered at the beginning of each week before the behavior testing. The analysis of body weight during the course of the experiment showed significant differences among genotypes (F (1,34) = 77,106, p<0,001, ω2=0,684, n=8-10/group) (see Table 4). It is possible to observe that over time, there was an increase in body weight in the WT groups. For TG groups, there were some weight variations over time, but no evolution of body weight gain, which is in accordance with previous descriptions for this model [45], (see Figure 19). Although there are no significant differences, mice with access to the running wheel have higher body weight in the last timepoint analysed, at 39 weeks of age.

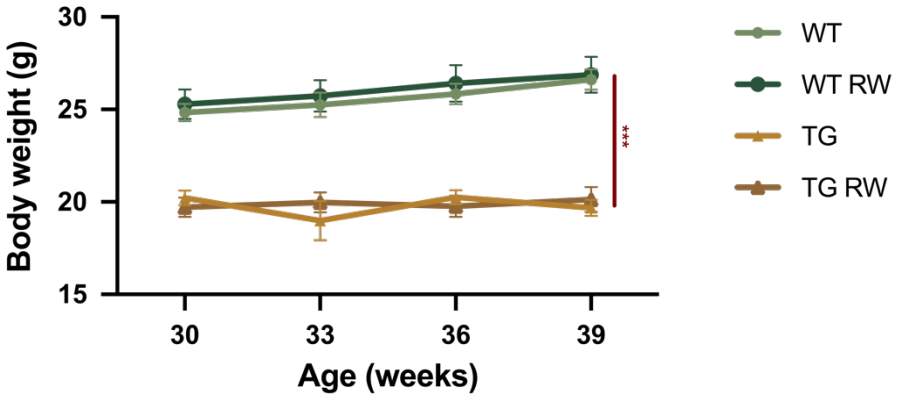

Figure 19 – Body weight during the course of the experiment - revealed significant differences between WT and TG animals (n= 8-10/group), but no effect of the RW was observed. Repeated Measures ANOVA and One-way ANOVA using Tukey HSD Post-Hoc analysis. Symbol represent the mean ± SEM of the different groups; *** represent the p⩽ 0.001.

Table 4 – Results of the statistical analysis of body weight during the experiment: multiple comparisons between groups, using One-Way ANOVA;*** represent the p⩽ 0.001 **.**

Samples	Weeks				
	30-39	30	33	36	39
WT vs. TG	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WT vs. TGRw	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WT vs. WTRw	0,960	0,938	0,976	0,926	0,993
WTRw vs. TG	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WTRw vs. TGRw	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
TGRw vs. TG	0,999	0,934	0,849	0,961	0,972

The analysis of body weight gain revealed significant differences over time regarding genotype, but also between TG groups (see Table 5). We can observe that in WT groups, mice gain weight throughout time, and in TG groups there is body weight gain and loss. In the case of the TG group without Running Wheel there is a higher variation compared to the TG with RW. Interestingly, it is also possible to observe that TG mice with RW showed a higher body weight gain (see Figure 20).

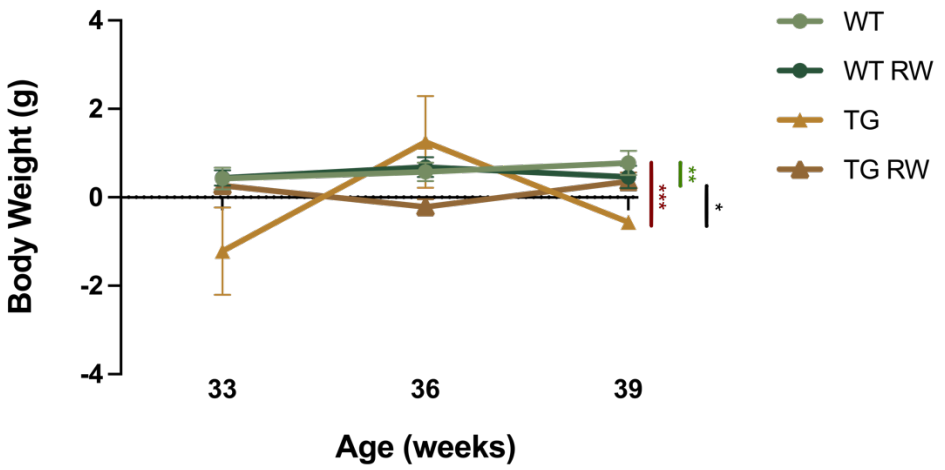

Figure 20 - Body weight gain showed significant differences between genotype, but also between TG groups (n= 8-10/group). Repeated Measures ANOVA and One-way ANOVA using Tukey HSD Post-Hoc analysis. Symbols represent the mean ± SEM of the different groups; *, **, *** represent the p⩽ 0.05; 0.01 and 0.001 respectively.

Table 5 – Results of the statistical analysis of the body weight gain: multiple comparisons between groups, using One-Way ANOVA; *, **, *** represent the p⩽ 0.05; 0.01 and 0.001 respectively**.**

Samples	Weeks				
	30-39	30	33	36	39
WT vs. TG	0,000 ***	-	0,147	0,831	0,001 **
WT vs. TGRw	0,002 **	-	0,997	0,783	0,593
WT vs. WTRw	0,928	-	1,000	0,999	0,737
WTRw vs. TG	0,000 ***	-	0,142	0,889	0,013 *
WTRw vs. TGRw	0,011 *	-	0,996	0,714	0,991
TGRw vs. TG	0,054 *	-	0,262	0,319	0,041 *

Physical exercise improved the balance of CMVMJD135 animals

In the beam walk test, mice needed to maintain their balance while traversing narrow beams to reach a safe dark box. By using the 12-mm square beam (the training beam), it was possible to verify an effect of RW on transgenic animals, in which the TGRW group achieved an identical performance to the WT groups (see Figure 21). Furthermore, at 36 weeks of age, TGRW performed significantly better when compared with TG mice, as they were able to fully complete the task, while TG mice failed to traverse the beam (see Table 6).

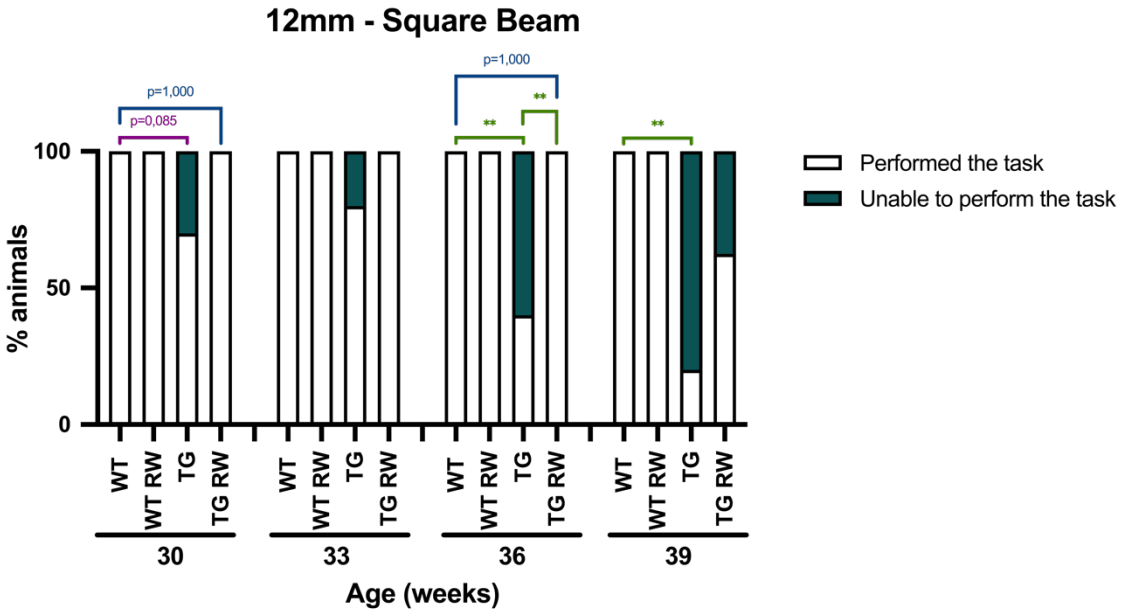

Figure 21 – The effect of RW on transgenic animals, evaluated by the 12mm- square beam test, which allows the evaluation of fine motor coordination and balance, showed significant differences between the genotype after 36 weeks of age, but not between the WT groups and the TGRW group *(n= 8-10/group)*.Statistical analyses using Independent – samples, Kruskal-Wallis test, using pairwise comparison of groups. The animal's performance was also analysed based on the following scores: Performed the task and unable to complete the task. Values are presented as percentage of animals (%); **represent the p⩽0.01.

Table 6 – Results of the statistical analysis of the square beam walk test: pairwise comparisons of groups. Independent – samples, Kruskal-Wallis test; ** represent the p⩽ 0.01.

Samples	Weeks			
	30	33	36	39
WT vs. TG	0,085	-	0,002**	0,001**
WT vs. TGRw	1,000	-	1,000	0,512
WT vs. WTRw	1,000	-	1,000	1,000
WTRw vs. TG	0,085	-	0,002 **	0,001 **
WTRw vs. TGRw	1,000	-	1,000	0,512
TGRw vs. TG	0,124	-	0,004**	0,307

When the difficulty was increased using a 11mm circle beam, the same result was verified, with an effect of RW on transgenic animals justified by the significant difference between TG groups (see Table 7). As the TG group showed a huge difficulty in performing the task (see Figure 22a), in the following time points a 28mm circle beam was used (see Figure 22b). Indeed, the observations were the same, as TGRW showed a better performance in the beam when compared to TG mice. Interestingly, TGRW mice presented a similar performance to WT mice, and only in the last timepoint analysed it was possible to detect some difficulty of the TGRW group in traversing the beam compared to the TG group.

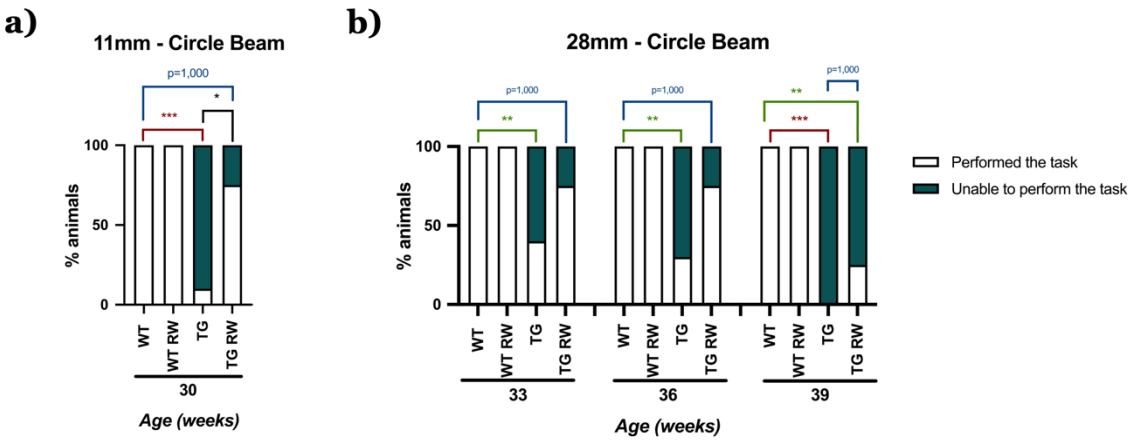

Figure 22 – Two types of circle beams were used to evaluate fine motor coordination and balance, revealing an effect of the presence of a RW in transgenic animals when the difficulty increases. % of animals that crossed a circle beam with 11-mm of diameter **(a)** and a circle beam with 28-mm of diameter **(b)**. The latency to cross the circle beam was recorded. Significant differences between genotype were found in circle beam (**a)** and **b)**), but not between the WT groups and the TGRW group *(n= 8-10/group)*. Significant differences were only observed at 39 weeks of age. Independent – samples, Kruskal-Wallis test, using pairwise comparison of groups. The animal's performance was also analysed based on the following scores: Performed the task and unable to complete the task. Values are presented as percentage of animals (%).; *, **, *** represent the p⩽ 0.05; 0.01 and 0.001 respectively.

Table 7 - Results of the statistical analysis of the square beam walk test: pairwise comparisons of groups. Independent – samples, Kruskal-Wallis test ; *, **, *** represent the p⩽ 0.05; 0.01 and 0.001 respectively.

Samples	Weeks			
	30	33	36	39
WT vs. TG	0,000 ***	0,007 **	0,002 **	0,000 ***
WT vs. TGRw	1,000	1,000	1,000	0,009 **
WT vs. WTRw	1,000	1,000	1,000	1,000
WTRw vs. TG	0,000 ***	0,007 **	0,002 **	0,000 ***
WTRw vs. TGRw	1,000	1,000	1,000	0,009 **
TGRw vs. TG	0,017 *	0,445	0,166	1,000

Horizontal exploratory activity is improved by the presence of Running-Wheels in CMVMJD135 mice home-cage

Horizontal movement, assessed by the number of squares travelled in an open arena, revealed that the groups with access to RW had a higher exploratory activity, while TG mice showed a decreased exploratory activity when compared to their WT-littermates (see Figure 23). Furthermore, the TGRW group showed a higher exploratory behavior than the TG group and, at some timepoints, there was no significant difference when compared to the WT group, suggesting a fully phenotype recovery (see Table 8).

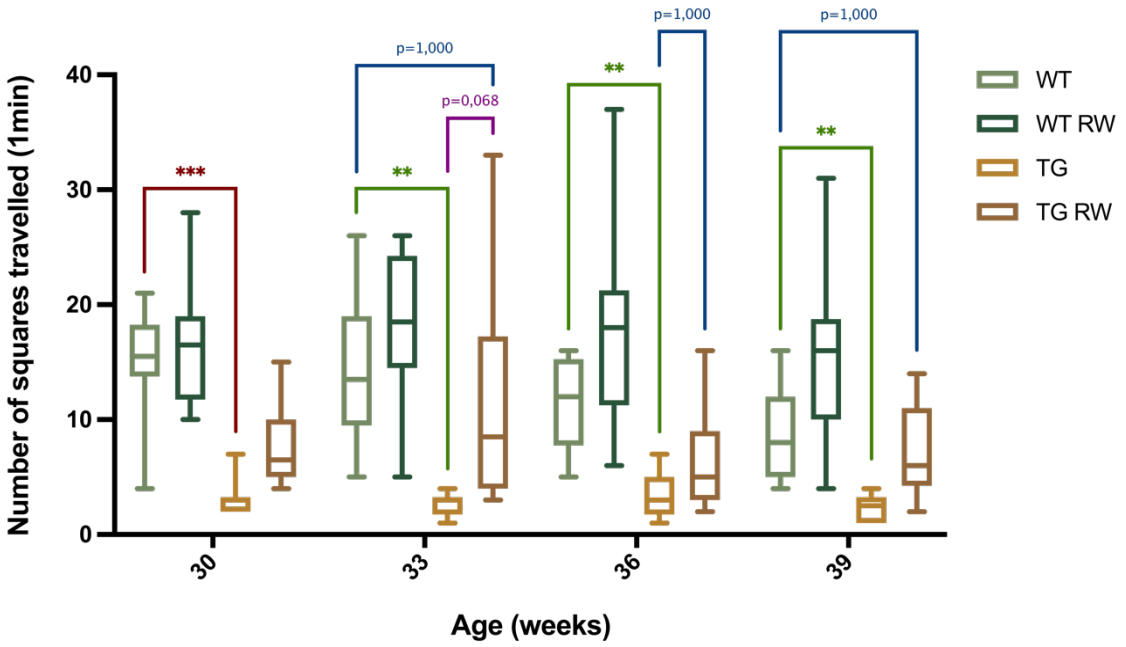

Figure 23 – The mice with access to RW had a higher exploratory activity in an open-squared arena. Significant differences were found for the horizontal movement performed in a squared arena, between WT groups and TG group, but the TGRW group showed a horizontal movement identical to the WT groups *(n= 8-10/group)*. Independent – samples, Kruskal-Wallis test, using pairwise comparison of groups. Values are presented as mean ± SEM.; **, *** represent the p⩽ 0.01 and 0.001 respectively.

Table 8 – Results of the statistical analysis of the horizontal exploratory activity test: pairwise comparisons of groups. Independent – samples, Kruskal-Wallis test; **, *** represent the p⩽ 0.01 and 0.001 respectively.

Samples	Weeks			
	30	33	36	39
WT vs. TG	0,000 ***	0,002 **	0,008 **	0,006 **
WT vs. TGRw	0,235	1,000	0,405	1,000
WT vs. WTRw	1,000	1,000	1,000	0,812
WTRw vs. TG	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WTRw vs. TGRw	0,090 *	0,576	0,014 *	0,206
TGRw vs. TG	0,404	0,068	1,000	0,106

Challenging tests to measure muscular strength showed no evident improvement by the RW in the CMVMJD135 mice

Loss of muscular strength is a very early and severe symptom observed in the CMVMJDQ135 mice. Thus, forelimb and hindlimb strength were also evaluated.

In the Hanging Wire Grid test, the four-limb muscular strength was evaluated. It is possible to verify a clear significant difference between genotypes (F (1,32) = 7452,127 p<0,001, ω2=0,996, n=8-10/group) (see Table 9 and Figure 24). In the WT groups there was no difficulty in complying with the test (as they hold on the grid for the maximum time of 120 seconds), which suggests that mice do not have impairments in

fine coordination and muscular strength. Regarding the TG groups, there was a clear loss of muscle strength that was aggravated over time. It is also possible to verify that access to RW did not imply a significant increase in muscle strength, although the TGRW group had a slightly higher latency than the TG group.

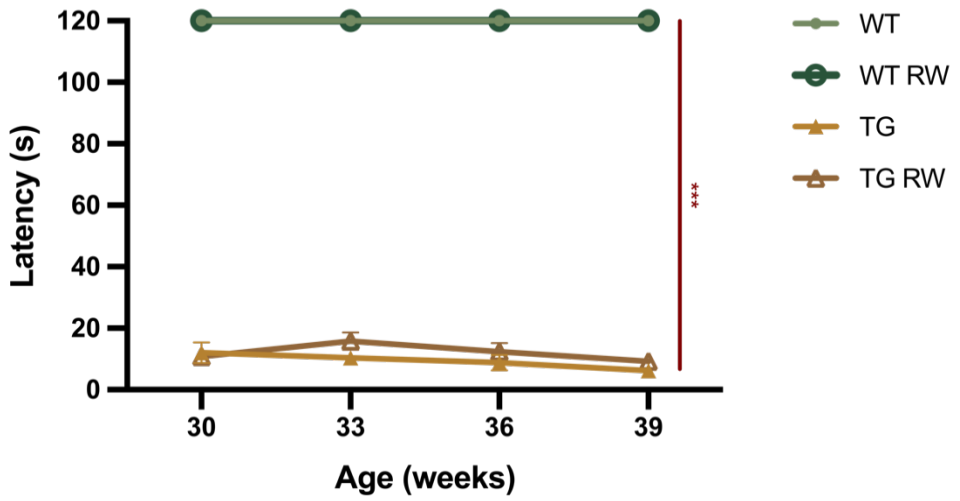

Figure 24 – The Running Wheel did not cause a significant increase in muscle strength, as evaluated by the hanging wire grid test. Significant interactions between genotype were found, more specifically between WT groups and TG groups. The TGRW group displayed a similar performance when compared to the TG group. Repeated Measures ANOVA and One-way ANOVA using Dunnett T3 Post-Hoc analysis. Each point represents the mean ± SD for the different groups; *** represents $p \leqslant 0.001$.

Table 9 - Statistical analysis of the hanging wire test: multiple comparisons between groups. One-Way ANOVA; *** represent the $p \leqslant 0.001$.

Samples	Weeks				
	30-39	30	33	36	39
WT vs. TG	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WT vs. TGRw	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WT vs. WTRw	-	-	-	-	-
WTRw vs. TG	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WTRw vs. TGRw	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
TGRw vs. TG	0,951	1,000	0,598	0,917	0,843

In the case of the forelimb muscular strength (measured by the Strength to Grab test), only significant differences were recorded in the last timepoints regarding genotype (see Figure 25 and Table 10). TGRW mice performed similar to TG animals, indicating no improvements by the presence of RWs.

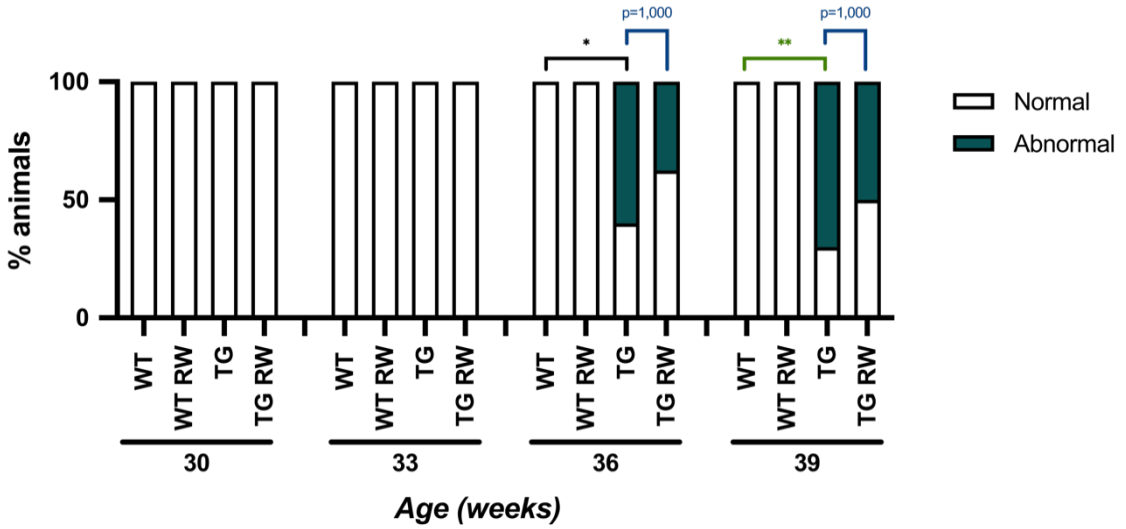

Figure 25 – Significant differences were found between the WT groups and the TG group, in the strength to grab test, after 36 weeks of age (n=8-10/group). The animal's performance was also analysed based on the following scores: Normal and Abnormal. Independent – samples, Kruskal-Wallis test, using pairwise comparison of groups. Values are presented as percentage of animals (%).; *, ** represent p⩽ 0.05; 0.010, respectively.

Table 10 - Statistical analysis of the strength to grab test: pairwise comparisons of groups. Independent – samples, Kruskal-Wallis test; *, ** represent p⩽ 0.05; 0.010, respectively.

Samples	Weeks			
	30	33	36	39
WT vs. TG	-	-	0,011 *	0,004 **
WT vs. TGRw	-	-	0,399	0,131
WT vs. WTRw	-	-	1,000	1,000
WTRw vs. TG	-	-	0,011 *	0,004 **
WTRw vs. TGRw	-	-	0,399	0,131
TGRw vs. TG	-	-	1,000	1,000

Additionally, in the Hindlimb Tonus test, both TG groups showed loss of hindlimb tonus resistance, confirmed by significant differences in genotype throughout the study (see Table 11). It should be noted that although there were no significant differences between the TG mice, the TGRW group revealed greater hindlimb tonus resistance as can be seen in Figure 26.

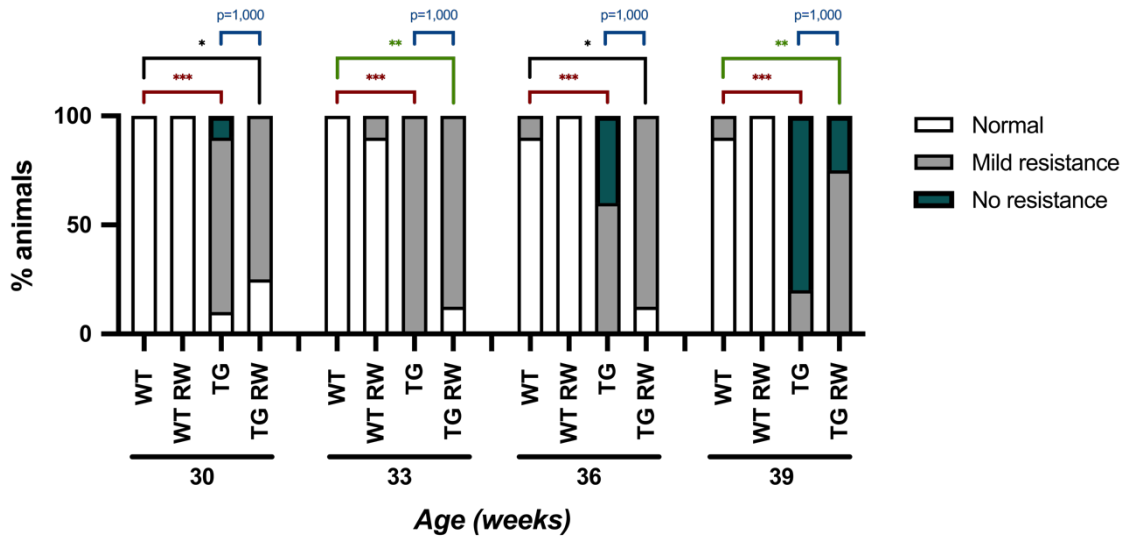

Figure 26 - The TGRW group revealed greater hindlimb tonus resistance, but there were no significant differences between the TG mice. Significant differences were found between the WT groups and the TG group (n=8-10/group). The animal's performance was also analysed based on the following scores: Normal, Mild resistance and No resistance. Independent – samples, Kruskal-Wallis test, using pairwise comparison of groups. Values are presented as percentage of animals (%).; *, **, *** represent the p⩽ 0.05; 0.01 and 0.001 respectively.

Table 11 - Statistical analysis of the hindlimb tonus test: pairwise comparisons of groups. Independent – samples, Kruskal-Wallis test; *, **, *** represent the p⩽ 0.05; 0.01 and 0.001, respectively.

Samples	Weeks			
	30	33	36	39
WT vs. TG	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WT vs. TGRw	0,012 *	0,002	0,010 *	0,004 **
WT vs. WTRw	1,000	1,000	1,000	1,000
WTRw vs. TG	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WTRw vs. TGRw	0,012 *	0,007 **	0,032 *	0,012 *
TGRw vs. TG	1,000	1,000	1,000	1,000

CMVMJD135 with access to Running-Wheels showed less severe hindlimb clasping

Regarding neurological reflexes, it was possible to observe significant differences between the WT and TG groups, where TG mice showed presence of hindlimb clasping (see Table 12). Interestingly, at 30 weeks of age, the TGRW animals showed significant less limb clasping when compared to TG mice, and, furthermore, no differences were observed between TGRW and WT mice. Throughout time, both TG and TGRW showed a worsening of this phenotype, nevertheless, TGRW always showed better neurological reflexes (see Figure 27).

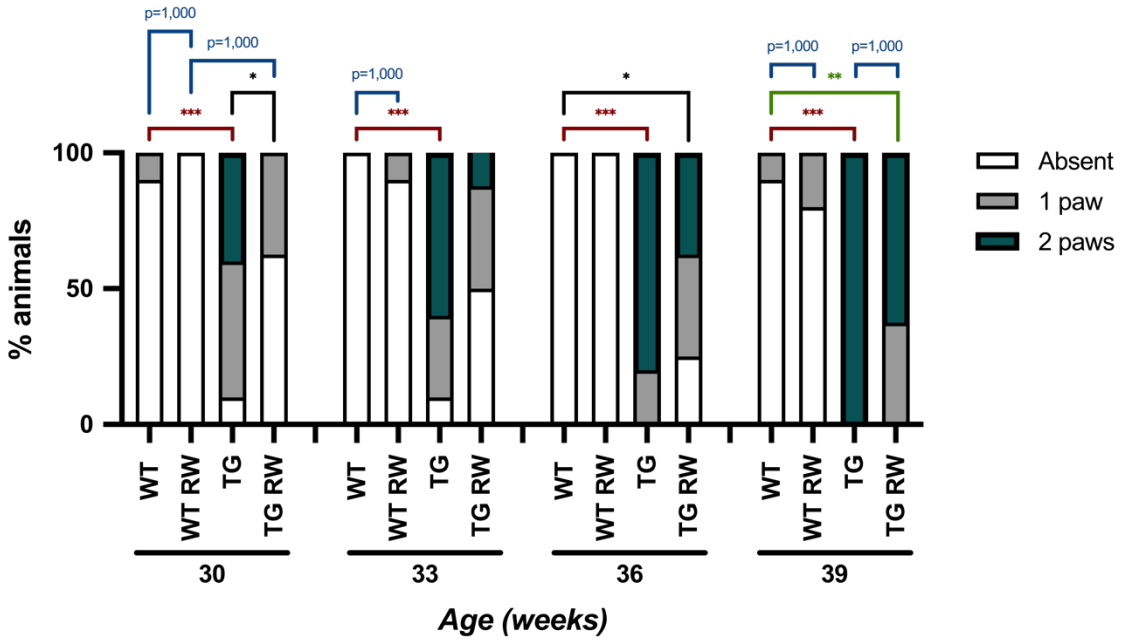

Figure 27 - The TGRW animals showed significant less limb clasping. Significant differences were found between the WT groups and the TG group (n=8-10/group). The animal's performance was also analysed based on the following scores: Absent, 1 paw and 2 paws. Independent – samples, Kruskal-Wallis test, using pairwise comparison of groups. Values are presented as percentage of animals; *, **, *** represent p⩽ 0.05; 0.01 and 0.001, respectively.

Table 12 – Results of statistical analysis of the clasping test: pairwise comparisons of groups. Independent – samples, Kruskal-Wallis test; *, **, *** represent p⩽ 0.05; 0.01 and 0.001 respectively.

Samples	Weeks			
	30	33	36	39
WT vs. TG	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WT vs. TGRw	0,892	0,339	0,029 *	0,003 **
WT vs. WTRw	1,000	1,000	1,000	1,000
WTRw vs. TG	0,001 **	0,001 **	0,000 ***	0,000 ***
WTRw vs. TGRw	1,000	0,706	0,029 *	0,007 **
TGRw vs. TG	0,054 *	0,211	0,874	1,000

CMVMJD135 animals showed mild gait improvement by physical exercise

Footprint patterns revealed the presence of footdragging from the beginning in TG mice. In Figure 28 and Table 13, it is possible to see the significant differences when comparing genotypes. Unexpectedly, WT animals showed some degree of footdragging, which can be justified by the fact that they perform the task very quickly and the adhesion of the paws to the paper is not the best due to the presence of ink on the paws. However, this observation does not rule out the possibility of footdragging over time. No differences were observed between TG and TGRW in this parameter.

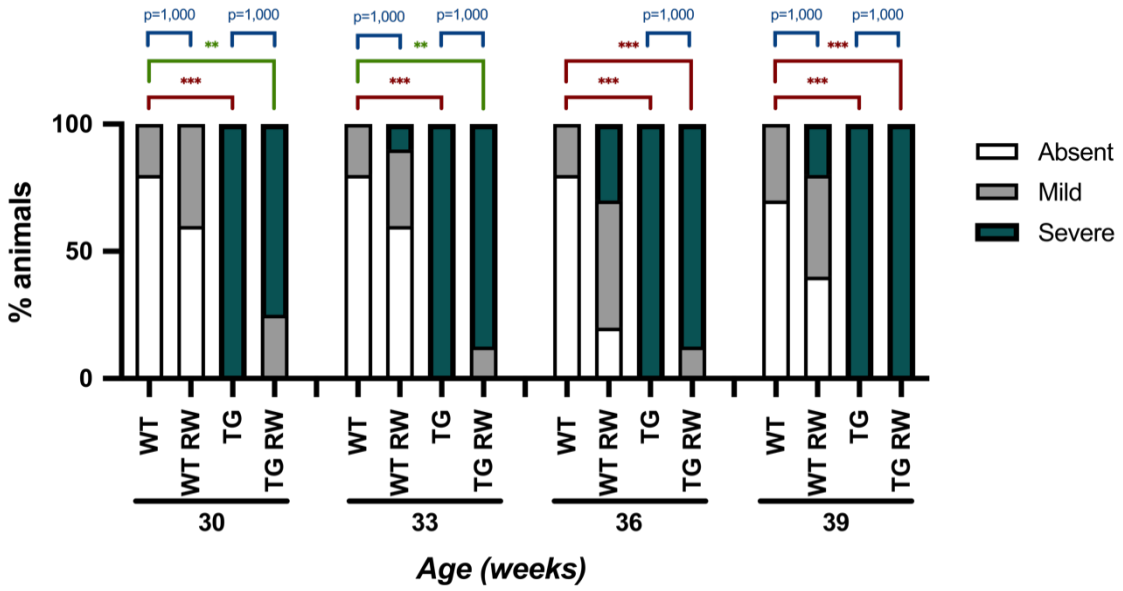

Figure 28 – TG mice showed the presence of footdragging from the beginning. Significant differences were found between the WT groups and the TG group (n=8-10/group). The animal's performance was also analysed based on the following scores: Absent, Mild and Severe. Independent – samples, Kruskal-Wallis test, using pairwise comparison of groups. Values are presented as percentage of animals (%); **, *** represent the p⩽ 0.01 and 0.001, respectively.

Table 13 – Results of statistical analysis of the footdragging test: pairwise comparisons of groups. Independent – samples, Kruskal-Wallis test; **, *** represent p⩽ 0.01 and 0.001, respectively.

Samples	Weeks			
	30	33	36	39
WT vs. TG	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WT vs. TGRw	0,001 **	0,001 **	0,000 ***	0,000 ***
WT vs. WTRw	1,000	1,000	0,202	1,000
WTRw vs. TG	0,000 ***	0,001 **	0,062 *	0,017 *
WTRw vs. TGRw	0,010 *	0,009 **	0,240	0,009 **
TGRw vs. TG	1,000	1,000	1,000	1,000

From the stride length measures, very intriguing results were observed. In addition to registering a significant difference regarding genotype (F (1,30) = 88,398 p<0,001, ω2=0,747, n=8-10/group), there was a significant difference between the WT groups, where WTRW animals showed a higher stride length (see Table 14). In the case of TG mice, the TGRW showed longer stride than the TG mice, which is statistically significant at 39 weeks of age. (see Figure 29).

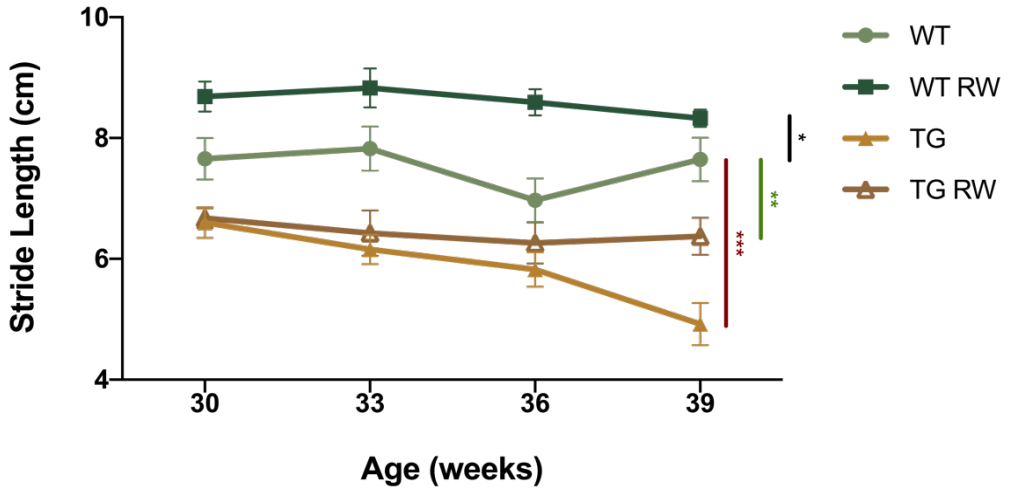

Figure 29 – At 39 weeks of age, TG animals with access to the RW showed a higher stride length. The TGRW group showed a statistically significant longer stride compared to the TG group, and it was possible to make the same observation between the WT groups (n=8-10/group). Repeated Measures ANOVA and One-way ANOVA using Tukey HSD Post-Hoc analysis. Symbols represent the mean ± SEM of the different groups; *, **, *** represent the p⩽ 0.05; 0.01 and 0.001, respectively.

Table 14 - Results of statistical analysis of the stride length test: multiple comparisons between groups. One-Way ANOVA; *, **, *** represent p⩽ 0.05; 0.01 and 0.001, respectively.

Samples	Weeks				
	30-39	30	33	36	39
WT-TG	0,000 ***	0,040 *	0,005 **	0,052 *	0,000 ***
WT-TGRW	0,001 **	0,074	0,034 *	0,390	0,034 *
WT-WTRW	0,025 *	0,039 *	0,150	0,002 **	0,373
WTRW-TG	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
WTRW-TGRW	0,000 ***	0,000 ***	0,000 ***	0,000 ***	0,000 ***
TGRW-TG	0,438	0,997	0,939	0,764	0,010 *

The activity of the mice in the home-cage 24/7: the DVC® robustly detected the CMVMJD135 mouse phenotype and the RW presence increased their activity

The spontaneous activity of the mice in the home-cage was recorded 24/7, but for the data analysis the night period was chosen, because it is the period of highest activity of the mice (see Figure 30a). We can observe that the WT groups have a higher activity in the home-cage compared to the TG groups, and this difference is statistically significant (see Figure 30b). It should be noted that mice with access to RW have higher levels of activity, and that in an assessment over time, there is no statistically significant difference between the WT group and the TGRW group (see Figure 30c).

Regarding the analysis of voluntary physical exercise practiced in the RW, it is possible to observe the difference in kilometres travelled between the two groups (see Figure 30d). In order to obtain higher reproducibility, only in the weeks in which there was no intervention by the experimenter to perform the behavior tests were analysed (see Figure 30e). Although it is possible to see a difference in exercise practiced in RW between the two groups, there is no statistically significant difference in the analysis of the average daily kilometers travelled (see Figure 30f).

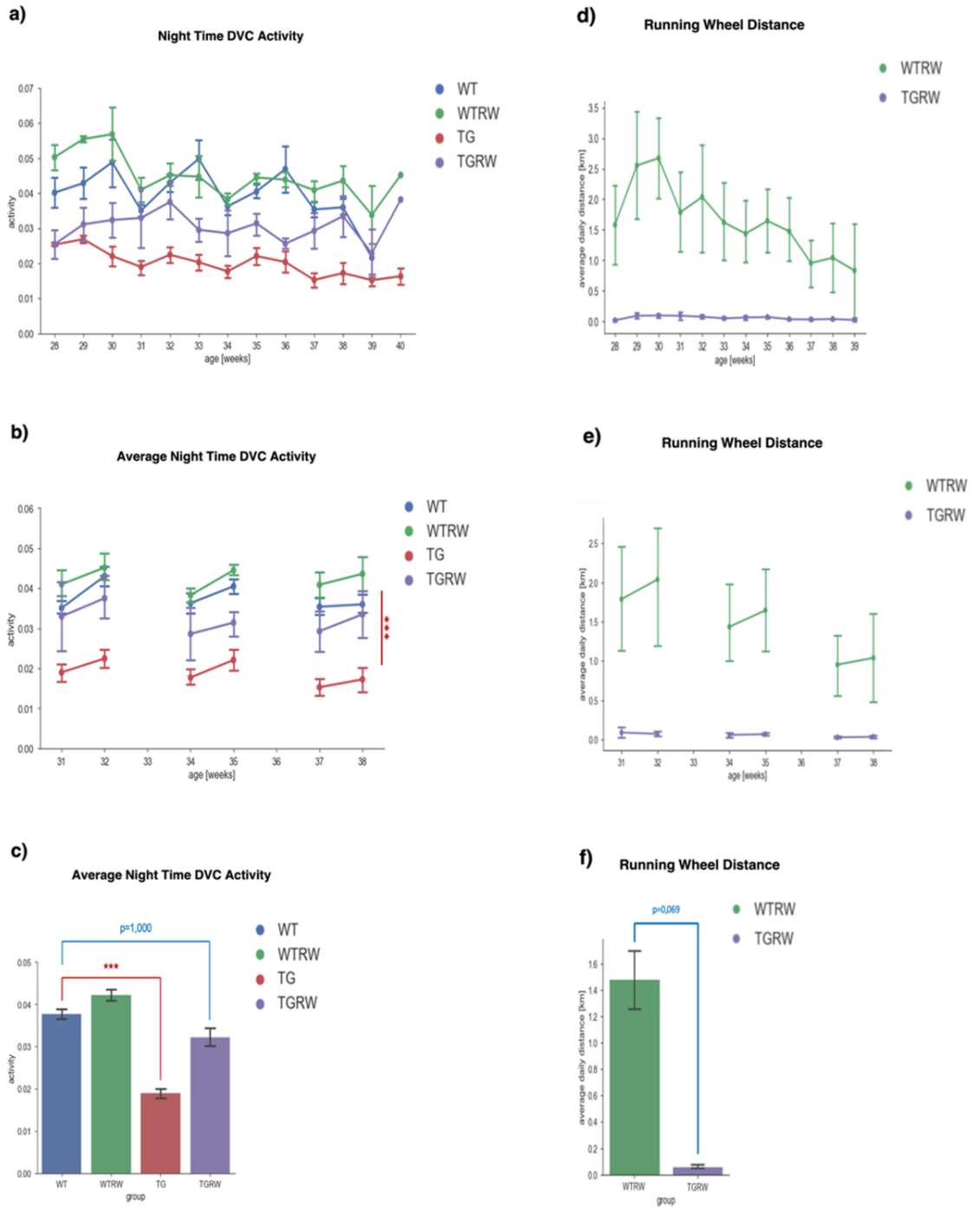

Figure 30 – The activity of the mice in the home-cage 24/7. a) Night time averaged activity registered by the DVC® between weeks 28 and 40; **b)** Average night time DVC® activity, excluding weeks in which behavioral tests were performed. WT mice perform the highest spontaneous activity; **c)**; TGRW mice showed a significant higher activity with respect to TG mice; **d)** Distance performed on the running wheel registered by the DVC® between weeks 28 and 40; **e)** Distance performed on the running wheel excluding weeks in which behavioral tests were performed; **f)** Average daily distance performed on the running wheel by the WTRW and TGRW groups. Even though presenting higher spontaneous activity then TG mice, TGRW run a very low distance on the running wheel when compared to WT mice. Repeated Measures ANOVA and One-way ANOVA using Bonferroni correction Post-Hoc analysis. Symbols represent the mean ± SEM of the different groups. *** represent the $p \leqslant 0.001$.

Chapter 5 – Discussion

As described above, MJD patients suffer from a progressive neurodegenerative disorder [3], [17], [18]. Depending on the degree of severity of the disease, it affects the quality of life of patients over time, as well as their average life expectancy differently [21], [22]. So far, there is no therapeutic options to treat or delay this disease, rather than symptoms relief medication [47]. Some characteristics of this disease, such as loss of motor coordination and muscle strength, are the main problems that lead to a functional decline and a decrease in physical activity [1]. In recent years, physical exercise has emerged as the most efficient and low-cost way for a healthier aging process, hence, it has the potential to be a good therapeutic strategy for age-related neurodegenerative diseases [90]. Different physical exercise protocols were administered to different models of neurodegenerative diseases, including polyglutamine diseases, and in general all of them showed a beneficial effect on symptoms and pathology [91]–[97]. In the case of Spinocerebellar Ataxia type 1 (SCA1), 4 weeks of a mild exercise regimen (in a fixed speed wheel) significantly increased the lifespan of the Atxn1154Q knock-in mice by increasing the levels of epidermal growth factor (EGF) and decreasing the levels of capicua (downstream of EGF signalling) and a known Atxn1 interactor [91]. Also, in Huntington's Disease, increased voluntary exercise by the running wheels availability in mice home cages lead to an improvement in motor and cognitive deficits, as well as neuropathology [92]. It is also described that in the case of Parkinson's disease, the practice of physical exercise can slow down the onset of symptoms and improve the general quality of life of the patients [48]. However, no study in MJD patients has yet been described.

Here, we used a fully automated rack system that allowed us to detect the progressive symptoms of the CMVMJD135 mouse model, and in addition, by the presence of a Running Wheel we were able to collect data on voluntary physical activity practiced within the home-cage. In order to better understand the data provided by the DVC®, a standardized battery of motor tests, already validated for the mouse model CMVMJD135 [86], [88], was performed.

In the present work, one of the major goals was to test the ability of the DVC® automated system to detect the disease progression of the CMVMJD135 mouse model of MJD. This model was previously described as having a robust and progressive phenotype over time, similarly to what is observed in MJD patients [45]. Additionally,

we also tested voluntary exercise as a potential therapy for MJD. For that, a running wheel was added to the mice home-cage. As the DVC® is still in some stages of development, we encountered some technical issues regarding the running wheels. These troubleshooting was also part of the present study.

Thus, we had to overcome such problems, which could imply changes in the animals' performances, until we reached a final version of the Running Wheel that would provide us with reliable data. The problems that were initially detected with the running wheels included the excessive noise produced during the running exercise, as well as the increased difficulty for the animals to make a turn in the wheel. These problems were related to the accumulation of dust on the running wheel, and to the consecutive material washings (wheels wear). Thus, to overcome these problems, new designs of running wheels were produced, and new running wheels had to be introduced in the cages to replace the defective ones. With this, the experimental design had to undergo several adaptations, and it was only possible to start the study at an already late stage of the disease. It should be noted that, although the data shown in this work comprises ages between 30 and 39 weeks of age, the animals had access to non-final Running Wheel versions prior to the one studied, since 6 weeks of age. That said, it is important to emphasize that the animals did not start physical exercise at a late stage, but from their first weeks of age.

An important feature of MJD is the CAG repeat instability [11], [34], [35]. Also, in this case, the CMVMJD135 model mimics the human condition as it presents this polyglutamine expansion instability, both when transmitting the mutant allele to the progeny or somatic mosaicism. The CAG repeat size is extremely important because it correlates positively with disease severity. Thus, while generating the experimental groups, it is important to control for the CAG repeat length. The length of the CAG repeat was not known at the time the transgenic animals were distributed among the different groups. This led to the fact that the animals in the TG group had involuntarily longer CAG repeat lengths, compared to the TGRW group (Figure 17a). Although, between the TG and TGRW groups, the statistical analysis did not show any statistically significant difference, to guarantee that the CAG repeat was not contributing to the differences observed, we analysed the performance of the mice in a motor coordination test, the swimming-pool test, according to the CAG repetitions (Figure 17b). It was possible to observe that the higher CAG repetitions did not directly imply worse performance of the mice. With this confirmation, we continued the study, as one should not expect the differences in CAG to have an impact on the other tests and parameters

analysed, as it didn't affect the swimming-pool test – an important test in to phenotype MJD mice models.

The behavioural assessment of CMVMD135 mice revealed the presence of several phenotypic abnormalities that gradually appear and aggravate throughout life. The deficits observed, such as loss of balance, motor coordination, loss of muscle strength, decreased locomotor and exploratory activity, and abnormal gait, were assessed using a standardized battery of tests. Each behaviour test performed was designed to assess the onset or evolution of the symptoms already described for the CMVMJD135 mouse model.

Throughout the study, the body weight of each animal was recorded. As expected, the WT groups increased their weight over time, with no significant differences between them. However, it is possible to observe that the WTRW group has higher body weight values compared to the WT group. This fact can be explained by the gain of muscle mass with the practice of physical exercise in the RW. In relation to transgenic animals, the body weight is, from the beginning of the study, lower relative to the WT groups. It is known from the literature that CMVMJD135 mice increase their body weight until a certain age, and after that point they do not gain weight, stabilizing their body weight. Here, and despite the oscillations recorded over time, the body weight has been constant and, interestingly, TG mice in the presence of the RW, showed a more stable body weight gain when compared to TG mice without RW, suggesting a protective effect in animal welfare by the RW.

Regarding the tests that aim to assess balance and motor coordination, it was possible to observe that access to RW has an influence on the animals' performance over time. The decreased balance and motor coordination, measured by the beam walk test, appeared earlier in the TG group, compared to the TGRW group, which maintained an identical performance to the WT group, without any significant difference, until 36 weeks of age, suggesting that the presence of the RW, delayed disease onset. Looking at the test that aims to understand the ability to swim (motor coordination), the TG animals demonstrate a greater latency to cross the water tank compared to the WT, registering a clear significant difference regarding the genotype, accordingly to what is described for this model. There was no statistically significant difference between the TG and TGRW group; nevertheless, it is possible to observe (Figure 19) that the TGRW group has a tendency towards a more consistent and lower latencies to traverse the water tank. Despite the fact that we could not accomplish the original experimental design of this study, due to the technical issues with the RWs, we were still able to

observe a tendency favouring a better performance of the TGRW. One must notice that the data presented here were analysed at very late stages of the disease development, where the phenotype and the pathology are fully established and yet a beneficial effect could be associated with the presence of RW. These results point for a great potential of physical exercise as a therapy for MJD. Accordingly, unpublished, and preliminary data from the group has shown a significant impact of exercise in the CMVMJD135. Two different cohorts of mice that were enrolled in different preclinical trials and different experimental designs, allowed to compare the degree of "physical exercise" by the bi-monthly or monthly repetition of the behavioral tests. Briefly, the "low exercised" transgenic animals were involved in a pre-clinical trial, in which animals were tested in a battery of motor tests every 4 weeks. The "high exercised" transgenic mice were involved in another pre-clinical trial, in which the animals were tested in a battery of motor tests every 2 weeks. At the same age, and although the "high exercise" animals also had a higher mean CAG repeat length than the "low exercise" group - 139 versus 131, and hence were more likely to be more severely affected, it was possible to observe that they performed better in the motor swimming test, taking significantly less time to traverse a 60 cm water tank. These experiments were run separately and not designed to verify the effects of exercise, yet its results seem to be in accordance with the results obtained in the present study.

Intriguingly, in the case of the WT groups, it is important to highlight the fact that the WT group showed a better performance than the WTRW group. This result suggests that access to RW does not have an overall positive impact in terms of performance for the WT mice. It was previously shown that mice under a high fat diet regimen (increased body weight), swam less than sedentary animals under a standard diet, suggesting body weight influence on swimming performance [98]. Therefore, and considering that WTRW groups showed an increased body weight compared to WT animals, this might be a factor contributing to their worst performance in the water tank.

Loss of muscular strength is the first and most severe phenotype observed in the CMVMJD135 mouse [45]. To date, several preclinical trials were performed in this mouse model and among many compounds tested (Reviewed in [99]), only creatine was able to improve muscular strength deficits. This was likely due to the ability of creatine to serve as an energetic pool for the cells, increasing muscular capacity [100]. Here, we showed that the TG group has an earlier and faster decrease in muscle strength compared to the TGRW group, suggestive of an increased muscular capacity by the presence of the RW. One can speculate that the physical exercise on the RW

provides a better functioning of the body, working the different muscles and can promote an increase in muscle mass / strength, leading to the differences we observed for muscular capacity.

An important novelty of the present work relies on the use of a fully-automatized system to detect mice daily activity in their home-cage without the interference of the experimenter. In fact, we used this system for the first time in a mouse model of MJD, and we were able to conclude that the DVC® system efficiently detects the motor deficits associated with the CMVMJD135 mouse model, by recording lower activity in TG animals when compared to their WT-littermates. Additionally, using the automated rack system - DVC® plus the RW, it was possible to find that the animals with access to the RW had higher spontaneous activity. The TGRW group did not present significant differences compared to the WT group, and, accordingly, the same was verified in the horizontal movement test. We can assume that access to the RWs may stimulate exploratory activity in mice and thus imply higher spontaneous activity.

Not surprisingly, the physical exercise practiced in RW by the TG animals was much less than in the WTRW group. Thus, it is possible that the beneficial effects observed by the presence of the RW in this study are also correlated with the fact that the animals had access to different versions of the RW since 6 weeks of age, even though the RW model provided at the beginning did not work properly, it still allowed some degree of voluntary exercise to the mice. These results are in accordance to several reports where physical exercise (mostly involuntary) showed to improve the phenotype of several animal models of neurodegenerative diseases [90], [101], [102]. Altogether, our results point for the practice of physical exercise as an important therapy (by itself or in combination with other therapeutic approaches) for MJD. In this line, it would be of extreme importance to further explore this hypothesis by performing a more detailed experimental design using the most adequate running wheels, as well as study the impact of this therapy in MJD pathology and, ultimately, studying the cellular/molecular mechanism(s) underlying this beneficial effect.

Chapter 6 – Conclusions and future perspectives

In this dissertation, we have shown that there is an influence of voluntary physical exercise in the motor dysfunction of a mouse model of Machado-Joseph disease (MJD), the CMVMJD135. The overall results showed a tendency for the Transgenic mice with access to the Running Wheel to present a better performance, in the behavioral tests that evaluate motor skills, when compared to Transgenic mice without Running Wheel.

The promising results in the behavioral tests as well as in the spontaneous activity automatically recorded in the DVC®, point to further and more extensive characterization of the effect of physical exercise in this mouse model. However, it is important to better understand the levels of exercise practiced by the mice on the Running Wheel available at their Home-cage.

Furthermore, the DVC® automated system may be of great importance to study other models of disease, being a complementary tool to animal behavioral testing. In this study we proved its efficacy in revealing different phenotypes of mice, showing the automatic activity screening can be used as a powerful tool to discriminate Wild Type from CMVMJD135 Transgenic mice.

Thus, in the near future, we propose to:

1) **Perform a new preclinical study using the final version of the RW.** Similar to what was presented in this dissertation, it would be very interesting to pursue this scientific question and evaluate the therapeutic effect of physical exercise in the CMVMJD135 by providing the RW at an early asymptomatic age and evaluating its effects on disease onset and progression.

2) **Determine the levels of physical exercise practiced.** Evaluate the "amount" of physical exercise practiced by TG mice, and correlate those with motor performance on standardized motor tests.

3) **Evaluate the effect of running in the neuropathology of MJD.** Considering that voluntary exercise led to a partial amelioration of the behavioural deficits in transgenic mice, it would be interesting to evaluate, for instance, whether it also elicited

changes at the neuropathological level, by assessing the amount of ataxin-3 positive inclusions, the number of ChAt-and Calbindin-positive neurons in the spinal cord and cerebellum, respectively, as well as the neuroinflammatory profile.

4) **Evaluate the physiological effects on muscles, in mice with RW.** Voluntary wheel running can result in robust endurance-like adaptation in skeletal and cardiac muscles. Considering the pre-existent muscular mass loss and reduction in quadricep skeletal muscle fibers in the CMVMJD135 mice (unpublished data), it would be interesting to evaluate, for instance, the skeletal muscle mass (quadricep, gastrocnemius, tibialis anterior, triceps), the changes in the fiber type composition of skeletal muscle, and the changes in cardiac mass and cardiac hypertrophy adaptation to exercise between the TG animals and TG animals with access to Running Wheel.

5) **Investigate the molecular mechanism(s) underlying physical exercise effects.** Physical exercise is known to enhance the expression of growth and neurotropic factors, such as BDNF, as well as to promote neurogenesis. Additionally, as mentioned above, it is also described to upregulate the levels of epidermal growth factor (EGF) and downregulate the levels of capicua. Thus, examining the levels of these molecules could give us an insight on the mechanisms behind exercise's beneficial effect.

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Supplementary Information

Supporting information: Towards Large Scale Automated Cage Monitoring

Towards Large Scale Automated Cage Monitoring – Diurnal rhythm and impact of interventions on in-cage activity of C57BL/6J mice recorded 24/7 with a non-disrupting capacitive-based technique

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Poster

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