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Faculdade de Medicina de Lisboa

Validation of an automated equipment for depression induction in a rodent model

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Dissertação especialmente elaborada para obtenção do grau de Mestre em Neurociências

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RESUMO

A depressão é uma doença psiquiátrica multidimensional que afeta cerca de 350 milhões de pessoas em todo o mundo. O uso de modelos animais é muito importante no contexto da investigação e desenvolvimento de novos medicamentos para tratar a depressão, na fase de ensaios pré-clínicos. No entanto, a criação de modelos animais de depressão válidos e eficazes tem sido um desafio para vários investigadores deste campo.

Os modelos mais usados são baseados nos seguintes critérios de validação: semelhança (face validity), homologia (construct validity) e predição (predictive validity), validados por Willner. O critério de semelhança diz respeito à capacidade do modelo de mimetizar os principais sintomas da doença. O critério de homologia tem em consideração a fundamentação teórica por detrás das características observadas e o critério de predição avalia a correlação com a eficácia do tratamento na clínica.

Há vários modelos animais de depressão, uns são baseados na exposição ao stress, outros em manipulações (bio)químicas, outros baseados em genética e há mesmo alguns derivados de lesões. Para estudos pré-clínicos os modelos baseados na exposição ao stress são os mais fidedignos porque permitem uma avaliação do fármaco num organismo, enquanto sistema integrado. Para além disso, o stress crónico moderado e imprevisível (uCMS) é um modelo amplamente utilizado pela sua similaridade etiológica com a doença humana e pelo preenchimento de todos os critérios de validação referidos. A principal fraqueza do modelo é a sua pouca reprodutibilidade entre laboratórios, muito provavelmente derivada da falta de padronização. Adicionalmente, trata-se de um modelo moroso, exigente do ponto de vista operacional e suscetível a variabilidade na execução do protocolo.

Para ultrapassar estes problemas, a empresa onde desenvolvi esta tese de mestrado, *Bn'ML – Behavior and Molecular Lab*, desenvolveu um equipamento capaz de executar o protocolo de uCMS de uma forma padronizada e automatizada. Esta empresa é uma start-up da Universidade do Minho que trabalha na avaliação de efeitos comportamentais e moleculares de compostos terapêuticos, em modelos animais de doenças psiquiátricas.

Este trabalho, desenvolvido no laboratório ICVS/3B's - onde está incubada a Bn'ML, teve 2 objetivos principais: o primeiro foi o acompanhamento e supervisão da construção do protótipo assim como o teste e melhoramento individual dos stressores adaptados; o segundo foi a validação do protocolo de uCMS adaptado num estudo piloto utilizando um pequeno número de animais.

No âmbito do primeiro objetivo concluímos a construção do protótipo (semelhante a uma rack) e conectámos este equipamento a um computador, o que permitiu a robotização de um processo que era integralmente executado pelo operador. De seguida reformulámos alguns dos stressores que fazem parte do protocolo de uCMS, para isso dividimo-los em três categorias principais: stressores totalmente automáticos, stressores parcialmente automáticos e stressores não automáticos. Os stressores totalmente automáticos foram introduzidos nas funções do equipamento - estes stressores são executados sem qualquer manipulação por parte do operador. Stressores parcialmente automáticos não são controlados pela rack mas a sua forma de implementação foi modificada de forma a ser adaptada a uma estrutura de mais fácil uso para o operador. Finalmente, stressores não automáticos refere-se aos que permaneceram iguais e portanto a sua execução foi exatamente igual à do protocolo original. Após provar que estes stressores adaptados desenvolviam a uma resposta ao stress similar aquela que ocorre em stressores originais (através do seu teste individual com animais), estes stressores foram integrados no equipamento para possibilitar o desenvolvimento de um protocolo completo, no formato de um estudo piloto.

Para o segundo objetivo, integrámos todas as adaptações ao protocolo original (uCMS) num estudo piloto, o que nos possibilitou atingir resultados úteis e originais no que diz respeito às abordagens técnicas e metodológicas. Os principais resultados obtidos foram a redução do espaço necessário para executar este protocolo, a poupança de tempo, a diminuição da intensidade de trabalho, uma exposição mais uniforme das caixas dos animais ao protocolo (menos variabilidade), assim como a redução da manipulação dos animais. Estas alterações podem contribuir para ultrapassar as conhecidas limitações do protocolo de uCMS.

Os resultados do estudo piloto foram avaliados para perceber se os critérios de validação propostos foram preenchidos. Para isso, um grupo de animais foi exposto ao protocolo automatizado (auCMS) e comparado com o grupo exposto ao protocolo manual (uCMS) e com o grupo controlo (CT). Alguns animais do grupo de auCMS foram tratados com um antidepressivo, a fluoxetina, para verificar a sua capacidade de reverter o fenótipo induzido. Os critérios de validação foram avaliados através de análises moleculares e celulares (critério de homologia), testes comportamentais (critério de semelhança) e eficácia do tratamento (critério de predição).

A nível molecular, os níveis de corticosterona mostraram uma disrupção do padrão de secreção desta molécula nos animais expostos ao auCMS, tal como foi observado com o grupo uCMS. Relativamente à morfologia dos neurónios do giro dentado do hipocampo, ambos os grupos stressados (uCMS and auCMS) apresentaram resultados inconclusivos no que diz respeito ao comprimento das dendrites e à complexidade dos neurónios do giro denteado dorsal. Apesar

da validação da componente molecular, o critério de homologia foi apenas parcialmente validado devido à falta de dados robustos na componente celular.

O critério de semelhança (face validity) foi avaliado através de testes comportamentais para as 3 dimensões (cognição, ansiedade e humor), conhecidas por estar afetadas nesta doença. Resultados da cognição e ansiedade apresentaram diferenças significativas entre o grupo controlo e o grupo auCMS, com pior desempenho deste último grupo. A dimensão do humor foi avaliada através de 3 testes diferentes: SPT e SDT (testes anedónicos) que apenas mostraram diferenças significativas entre o grupo CT e o grupo uCMS; e pelo FST (teste de desamparo aprendido) que mostrou diferenças significativas entre o grupo controlo e o grupo auCMS. Estes resultados, pouco claros, podem estar relacionados com as dificuldades metodológicas deste tipo de testes. Apesar destas inconsistências, é possível alegar uma validade deste critério, visto que todos os testes comportamentais apresentaram piores desempenhos do grupo auCMS quando comparado com o grupo CT, sendo que naqueles onde não há estatística significativa existe uma tendência nesse sentido.

Por último, uma parte dos animais do grupo auCMS foram tratados com fluoxetina e expostos aos mesmos testes comportamentais. Apesar de nem todos atingirem a validade estatística, os resultados mostram uma melhoria do grupo tratado em todos os testes, o que poderá ser considerado como um preenchimento do critério de predição.

Para além destes critérios, Belzung introduziu outro conceito que pretende validar as estirpes usadas. O nosso estudo também teve em conta este parâmetro, uma vez que a nossa escolha recaiu nos *Wistar Han* – uma estirpe consensualmente aceite para estudar a depressão.

No geral, tendo em conta os critérios de validação analisados os resultados revelaram-se promissores visto que a maioria dos resultados do protocolo automatizado revelaram uma resposta similar ao manual e divergente dos controlos.

É importante não esquecer que este estudo piloto produziu resultados preliminares baseados num pequeno número de animais, o que levanta a necessidade de repetir a experiência para confirmar os resultados observados. Outra limitação está relacionada com a natureza em si de um estudo piloto, como foi o primeiro estudo desenvolvido com o equipamento algumas necessidades de aperfeiçoamento foram identificadas. Tal como esperado, ocorreram algumas falhas do equipamento assim como erros no protocolo programado; estas questões foram prontamente resolvidas mas ainda assim estes problemas podem ter influenciado a indução de stress nos animais e pode ter sido a causa para algumas alterações na aquisição do fenótipo.

Apesar de este trabalho ter sido desenvolvido para estudar a depressão através de um protocolo de exposição a stress crónico, o equipamento apresentado pode ser usado para outras doenças uma vez que este é capaz de desenvolver outro tipo de protocolos.

Nós acreditamos que esta inovação permitirá obter modelos animais mais robustos e fidedignos. De facto, modelos animais viáveis são cruciais para a investigação em ciências da saúde, particularmente para a melhoria das abordagens pré-clínicas atuais. Considero assim, que se deu um passo importante para o progresso no campo da investigação pré-clínica.

PALAVRAS-CHAVE: depressão, modelo animal, validação, stress

ABSTRACT

Depression is a multidimensional psychiatric disorder that affects around 350 million people worldwide. In order to study new treatment approaches for this disease it is of major importance to use animal models. However, the generation of valid and effective animal models of depression has been a challenging task for many researchers that work in this field.

The mostly used models are based on the following validation criteria: face, construct and predictive, validated by Willner. Face validity is whether the model mimics the core symptoms of the disease, construct validity takes into account the theoretical rational behind the features observed and predictive validity evaluates its correlation with treatment efficiency in the clinics.

There are several animal models of depression, some are based on stress exposure, others on (bio)chemical manipulations, others are models based on genetics or even derived from lesions. For pre-clinical studies the models based on stress exposure are the most reliable because they allow to evaluate the effects of a drug on an organism, as an integrated system. Furthermore, the Unpredictable chronic mild stress (uCMS) is a widely used model, based on stress, mainly due to its etiological similarity with the human disease and also for the fulfilment of all the validation criteria mentioned before. The main weakness of this model is its reduced reproducibility between laboratories, most likely to derive from the lack of standardization. Additionally, this model is very time-consuming, demanding from the operational point of view and susceptible to variability in protocol execution.

In order to overcome these issues, the company where I performed this master thesis, Bn'ML – Behavior and Molecular Lab, developed an equipment capable of performing the uCMS protocol in a standardized and automated manner. This company is a start-up from the University of Minho that works in the evaluation of behavioural and molecular effects of therapeutic compounds, in animal models of psychiatric diseases.

This work was developed at the ICVS/3B's laboratory, where B'nML is incubated and had two main goals: the first was the accompaniment and supervision of the prototype construction as well as to test and improve the adapted stressors, individually; the second was to validate this protocol adaptation in a pilot study with a small cohort of animals.

Within the scope of the first objective, we completed the prototype construction (similar to a rack) and connected this equipment to a computer, which allowed the robotization of a process that was fully executed by the experimenter. Then, we reformulated some of the actual

stressors that are part of the uCMS protocol, for that we divided them into three main categories: total automated stressors, partial automated stressors and non-automated ones.

Total automated stressors were introduced in the equipment functions –these stressors are performed without any manipulation of the experimenter. Partial stressors are not controlled by the rack but their way of being implemented was modified in order to adapt to a more user-friendly structure. Finally, non-automated stressors refers to the ones that remained unchanged and so their execution was exactly the same as in the original protocol. After proving that these adapted stressors could lead to a similar stress response when compared to the original stressors (through their individual test with animals), these adapted ones were integrated into the equipment to allow the performance of a complete protocol, in a pilot study format.

For the second objective, we integrate all the adaptations to the original procotol (uCMS) in a pilot-study, which enable us to reach useful and original outcomes with regard to technical and methodological approaches. The main outcomes obtained were a space-demanding reduction, saving time, a decrease in labour-intensity work, a more uniform exposure of the cages to the protocol (less variability) as well as a reduction of experimenter manipulation of the animals. These alterations may contribute to overcome some known limitations of the uCMS protocol.

The results from the pilot study were evaluated to understand if the proposed validation criteria were fulfilled. For that, a group of animals was exposed to the automated protocol (auCMS) and compared to a group exposed to the manual protocol (uCMS) and to the control group (CT). Some of the animals from the auCMS group were treated with an antidepressant, fluoxetine, to verify its ability to revert the induced phenotype. The validation criteria were assessed through molecular and cellular insights (construct validity), behavioural tests (face validity) and treatment efficacy (predictive validity). Molecular findings from corticosterone levels showed a disruption of the circadian regulation of animals exposed to auCMS, like it was observed with the uCMS group. Regarding neuronal morphology in the hippocampal dentate gyrus (DG), both stressed groups (uCMS and auCMS) showed unclear results in dendritic length and in complexity of dorsal DG neurons. Despite the validation of the molecular component, construct validity can only be partially validated due to the lack of robust results from cellular findings.

Face validity was evaluated through behavioural tests for the 3 dimensions (cognition, anxiety and humor), known to be affected in this disease. Results from cognition and anxiety show significant differences between control group and auCMS group, with a worse performance of this last group. Mood dimension was assessed through 3 different tests: SPT and SDT (anehonic tests), in which we only observed statistical differences between the CT and uCMS groups; and

the FST (learn-helplessness test) that showed statistical differences between the CT and auCMS groups. These unclear results can be related to methodological difficulties of this type of tests.

Despite this inconsistence it is possible to claim a validation of this criteria since all the behavioural tests showed a worse performance in the auCMS group when compared to CT ones, and in those where there is not a statistical significance it exists a tendency in that way.

Lastly, some animals from the auCMS group were treated with fluoxetine and allowed to perform the same behavioural tests. Although not all animals reached statistical differences, results showed an improvement of the treated group in all the tests, which may be considered a fulfilment of the predictive validity.

Apart from these criteria, Belzung introduced another concept that intends to validate the strains used. Our study also took this parameter into account since our choice relapsed on *Wistar Han* – a strain that is consensually accepted to study depression.

Overall the validation criteria evaluated showed promising results since the majority of them indicate a similar response to the manual protocol and divergent from the control ones.

Importantly, this pilot study produced preliminary results based on a small number of animals raising the need to repeat the experiments in order to confirm the results observed. Another limitation is related to the nature of a pilot study itself, as it was the first study developed with the equipment and some improvement needs were identified. As it was expected, some failures of the equipment occurred as well as errors in the programmed protocol; these issues were promptly resolved, however, these problems may have influenced the animals stress induction and could have been a cause for some alterations in the phenotype acquisition.

Although this work has been developed to study depression through a protocol of chronic stress exposure, the stated equipment can be used for other diseases since it is capable of performing other types of protocols. We believe that this innovation will allow developing more robust and reliable animal models. In fact, viable animal models are crucial to health sciences research, particularly to the improvement of the actual pre-clinical approaches. I consider that an important step toward progress in the field of preclinical research has been made.

KEY-WORDS: depression, animal model, validation, stress

ABBREVIATIONS LIST

ACTH – Corticotropic hormone

AD – Antidepressant

AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

auCMS – Automated Unpredictable Chronic Mild Stress

BDNF – Brain-derived neurotrophic factor

BrdU – Bromodeoxyuridine

CNS – Central Nervous System

CORT – Corticosterone

CRF – Corticotropin-Releasing Factor

CRH – Corticotropin-releasing hormone

CT – Control

CUS – Chronic Unpredictable Stress

DCX – Doublecortin

DNA – deoxyribonucleic acid)

DG – Dentate Gyrus

DSM – Diagnostic and Statistical Manual of Mental Disorders

FLX – Fluxoxetine

FST – Forced Swimming Test

GABA – Gamma-AminoButyric Acid

GAD – Generalized anxiety disorder

GC – Glucocorticoid

GFAP - Glial fibrillary acidic protein

GR – Glucocorticoid receptor

HPA – Hypothalamic-Pituitary-Adrenal

KO – Knock-Out

LTM – Long Term Memory

MAOI – Monoamine Oxidase Inhibitor

MD – Major Depression

MDD – Major depressive disorder

MDE – Major depressive episode

MR – Mineralocorticoid receptor

NA – Non-Automated

NAc – Nucleus Accumbens

NMDAR – N-metil D-Aspartat receptor

NOR – Novel object recognition

NPC – Neural Progenitor Cells

NSF – Novelty supressed feeding

OCT – Optimal cutting temperature compound

PA – Partial Automated

PBS – Phosphate-buffered saline

PFA – Paraformaldehyde

PFC – Prefrontal cortex

PNS – Peripheral Nervous System

SEM – Standard Error of Mean

SDT – Sweet Drive Test

SPT – Sucrose Preference Test

SGZ – Subgranular Zone

SNRI – serotonin-norepinephrine reuptake inhibitor

SSRI – selective serotonin reuptake inhibitor

STM – Short Term Memory

STAR*D – Sequenced Treatment Alternatives to Relieve Depression

SGZ – Subgranular zone

SVZ – Subventricular zone

TA – Total Automated

TCA – Tricyclic antidepressant

uCMS - Unpredictable Chronic Mild Stress

VTA – Ventral Tegmental Area

FIGURE INDEX

PHOTO INDEX

TABLE INDEX

INDEX

1. INTRODUCTION

1.1 Depression

1.1.1 Global impact

Major depressive disorder (MDD) is a highly prevalent mood disorder, known to affect around 350 million people worldwide(1). According to the World Health Organization, MDD will be the second leading cause of disability worldwide by 2020. This disorder affects more women than men and it often appears at a young age(2,3). Moreover, this disease affects various functioning areas like social, professional or occupational, posing a tremendous burden on society.

1.1.2 Diagnosis

According to Diagnostic and Statistical Manual of Mental Disorders (DSM)-V, MDD is defined by, at least, one major depressive episode (MDE) as well as the absence of mania and hypomania(4). Nine symptoms can be present in an MDD patient: loss of interest or pleasure in usually pleasurable situations or activities (anhedonia), depressed mood, change in appetite and weight, loss of energy, less concentration, changes in sleep patterns, guilty feelings or worthlessness, psychomotor retardation or agitation and suicidal ideation. To be considered a MDE, five of these nine symptoms must be present during the same 2-week period, being depressed mood or anhedonia one of them. During this 2-week period the required frequency can vary by symptom but most of the times it needs to be present almost every day (4).

MDD can be considered a multi-dimensional psychiatric disorder because it often covers impairments in different behavioural domains, including cognition, anxiety and mood (5). Indeed, generalized anxiety disorder (GAD) seems to appear more nosologically related to MDD than previously thought due to high comorbidity between mood and anxiety disorders (6). Cognitive impairments have also been commonly associated with MDD, particularly executive dysfunction(7).

1.1.3 Treatment

Antidepressants are the first line treatment for depression. Still, many patients do not benefit from currently available antidepressants. According to START*D (Sequenced Treatment Alternatives to Relieve Depression) trial, which was an interesting study that gathered data about the effectiveness of antidepressant drugs treatment in MDD patients, only 28-33% of the patients remitted after the first antidepressant treatment(8). This trial was the largest and longest study ever conducted to evaluate depression treatment(9). This rate of remission is quite low for a disease with such prevalence, reflecting the need for the development of new antidepressants.

Antidepressants may be divided in several classes. First-line antidepressants to treat MDD are selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) because they are associated with a better safety profile and tolerability. SSRIs include fluoxetine, sertraline, paroxetine, fluvoxamine, citalopram and escitalopram whereas SNRIs encompass venlafaxine and duloxetine.

Tricyclic antidepressants (TCAs) are other class of antidepressants which inhibit the reuptake of both serotonin and norepinephrine by blocking each transporter. However they are not specific and they also block other receptor sites (like histaminic, cholinergic, and α1-adrenergic). These are recommended as second-line antidepressants while monoamine oxidase inhibitors (MAOIs) are third-line antidepressants due to their increased number of side effects (10,11).

In persistent and severe cases, where antidepressants fail or produce insufficient response, combination of antidepressants are a frequently used strategy or, even its augmentation by the use of other drug classes like antipsychotics.

In particular cases, non-medication treatments are also chosen, including deep brain stimulation, electroconvulsive therapy and vagus nerve stimulation (12).

1.1.4 Aetiology

Depression is thought to result from the interplay between genetic and environmental factors(13,14). Genetic contributions are relevant to the onset of depression, however, heritability is only moderate (40% to 50%) and as such, depression is not simply considered a genetic disorder(15). Taking into account that around 60% of the factors involved in depression aetiology are not explained by genetic variability, environmental features were shown to play a crucial role in depression(16).

Stress is a widely known environmental precipitating factor for this disease(17–19). However, not all stress types are maladaptive (20). Many factors are used to categorize stress;

the effects of stress depend on the neurodevelopment stage (for example childhood adversity is a major risk factor for depression), the intensity and duration, the nature, the predictability and controllability (21). As such, stress within specific contexts or exceeding a certain intensity and/or duration can affect physiological and behavioural homeostasis, leading to maladaptive responses(22). Additionally, individual susceptibility, i.e. the way different individuals cope with stress is highly variable, depending on (epi)genetic elements. Understanding the mechanismsand the conditions for these variations is crucial to improve knowledge on the etiopathogenesis of neuropsychiatric disorders (21,23).

Among all the stress types, chronic stress has a leading position within environmental precipitating factors contributing to the development of MDD. As I will explain next, hypothalamic-pituitary-adrenal axis (HPA-axis) deregulation is one of the links that I will approach to explain this relationship between stress and depression.

1.1.5 Pathophysiology of Depression

The Central Nervous System (CNS) is responsible for the processing of both external and internal inputs and for adjusting responses according to possible changes or stimuli. Neuroimaging and postmortem studies in depressed patients have revealed changes in several brain regions. Structural and functional alterations in the prefrontal cortex (PFC) and hippocampus can explain the cognitive alterations usually observed in patients, like memory impairments, hopelessness or suicidal ideation. Amygdala, as well as related parts of the striatum (mainly ventral tegmental area and nucleus accumbens), are involved in the reward responses and in mediating aversive stimulus; these structures are known to be affected in depression since hedonic deficits, anxiety and decreased motivation is often seen in depressed patients(24).

In summary, neural circuitry pathways involved in emotion, reward response and executive function are impaired in this disease(12). In depression, it is possible to observe the disruption of a wide variety of systems that will be next briefly described:

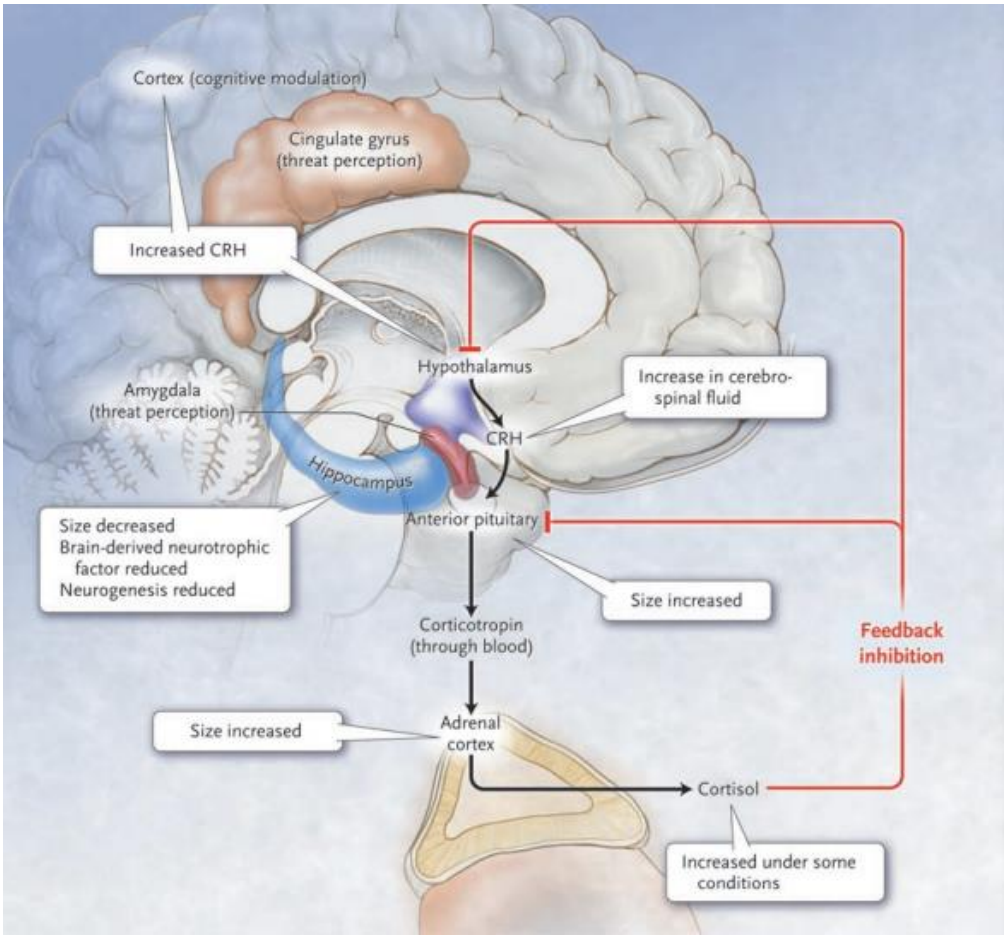

Figure 1. The Hypothalamic-Pituitary-Adrenal axis in Depression (25)

1.1.5.1 Neuroendocrine system

The Hypothalamic-Pituitary-Adrenal (HPA) axis controls glucocorticoids (GCs) release, a system deeply involved in stress response and in depression.

Stress is perceived in the cortex and this sensory information is transmitted to the hypothalamus where corticotropin-releasing factor (CRF) is released. This results in the secretion of the corticotropic hormone (CRH) from the anterior pituitary which will, in turn, stimulate the release of GCs from the adrenal cortex (cortisol in humans and corticosterone in rodents)(12). In fact, around 50% of depressed patients show elevated cortisol levels in the plasma and CRH in the cerebrospinal fluid(25). These GCs will activate the HPA axis through the binding to high-affinity MR (mineralocorticoid) and low-affinity glucocorticoid receptors (GR) – highly expressed in the hippocampus. The physiological regulation of this axis occurs by negative feedback triggered when GCs bind to their receptors.

In depressed patients, there is an hyperactivity of the HPA axis manifested by the increase of CRF and reduced feedback inhibition of the axis(12). This loss of the negative feedback loop is explained by a decrease of corticosteroid receptors in the hippocampus and PFC, also responsible

for the negative regulation of the HPA axis. As a consequence, this will lead to a persistent elevation of GCs secretion(22) (**Fig 1**).

Importantly, genetic studies also found a correlation between the genes encoding proteins involved in the regulation of the HPA axis and some variables related to the severity of the disease and its response to antidepressants(26).

1.1.5.2 Neuroimmune system

The neuroimmune system is associated with neuroinflammation and the neuroendocrine system, known to be affected in depression(27). Indeed, patients of infectious and autoimmune diseases often show depressive symptoms that are reverted with antidepressants(25). The other way around seems also to be true as MD patients have increased levels of the proinflammatory cytokines, tumor necrosis factor-α and interleukin-6(28), which were shown to affect the HPA axis and monoamines expression(25).

Similarly, chronic mild stress in rodents triggers the production of inflammatory cyto kines with an increase of interleukin-1β, tumor necrosis factor-α, interleukin-6 and interleukin-4 expression(29). Cytokine changes may be secondary to the stress associated with the illness and may not be related to the mood disturbance per se. In fact, cytokine elevations are most predominant in severe depression(30). Some explanations are beginning to emerge, suggesting that this elevation in cytokine levels may contribute to some aspects of the atypical symptomatology, including decrease in sex drive, increased sleep and muscle fatigue, which are well documented effects of proinflammatory cytokines.

1.1.5.3 Neurotransmission

A decrease **monoaminergic neurotransmission** was proposed as a model to explain the pathophysiology of depression. This proposal was based on the knowledge that mono amine-based agents are potent antidepressants – strong predictive validity(31). Indeed, norepinephrine and serotonin have critical roles in the mechanisms of action of several pharmacological treatments. However, serotonin and norepinephrine depletion does not induce depressive symptoms in healthy individuals(32).

The cause of depression is far from being a simple lack of central monoamines. Some results suggest that pre-synaptic monoaminergic receptors (that modulate monoamine release) have a reduced sensitivity with depression. Also, second-messengers of the signalling cascade of serotonergic and noradrenergic systems were shown to have a reduced functioning, which may impair neurotransmitter activity even without changing monoamine levels or receptor numbers.

As a result, caution must be taken when associating depression with a direct reduction in monoamine neurotransmitters(25,31).

Apart from monoamines, other neurotransmitters seem to be altered in depression, specifically **glutamate** which is the major excitatory neurotransmitter in the brain. A post-mortem study in brain tissue showed an increase in glutamate levels in the frontal cortex of MDD individuals and a decrease of plasma glutamate levels after antidepressants treatment(33). Contrary, other studies show increased levels of this neurotransmitter in the occipital cortex(25) and decreased levels in the prefrontal cortex of depressed patients(34), these inconsistent results need to be clarified in future studies.

Additionally, some studies show reduce expression of excitatory aminoacid transporters (EAAT1 and EAAT2) as well as glutamine synthetase (convert glutamate to glutamine) within glia in several brain regions of MDD patients. In line with this, a role of glia was reported with impairments glutamate uptake and metabolism(22,33).

Animal models support this findings, acute stress exposure induced an increase of extracellular glutamate in the hippocampus, amygdala and PFC. Repeated restraint stress lead to a reduction in AMPA-R and NMDA-R mediated synaptic currents in the PFC(22,33). Although, most of the animal findings support the idea that GC induces the enhancement of excitatory transmission there are some gaps between stress paradigms outcomes and their relationship with this NT that must be clarified (33).

Supporting the importance of this NT in depression, ketamine has appear as a putative innovative antidepressant. Actually, drugs that target the glutamate system must be deeply studied because they could bypass the typical delay of action of monoaminergic drugs (33). Ketamine, one of the most common NMDA antagonists, this drug is also an activator of AMPA receptors and an agonist of the D2 receptor of dopamine is an example of that(35). It was shown that a single subanesthetic dose of this drug was able to induce rapid and sustained antidepressant efficacy in depressed and treatment-resistant patients. In chronic stress rodent models, ketamine showed antidepressant-like properties, promoting an increase in synaptic connectivity and reversing the neuronal atrophy and behavioural deficits(36). Recent studies also suggest that antidepressant-like effects of NMDA antagonists depend on the enhancement of AMPA-R activation, which increases expression of BDNF and stimulates neurogenesis(33).

Another neurotransmitter that has been under study in depression is **GABA**, the main inhibitory neurotransmitter of the brain. Alterations of the GABAergic system in depression are not well understood yet(36,37). Some studies in MD patients reported reduced levels of GABA in the plasma, occipital cortex, PFC and cerebrospinal fluid. Unmediated depressed individuals also showed decreased protein and mRNA levels. This was also reported for PFC as well as decreased

protein and mRNA levels of glutamic acid decarboxylase (GAD) 67 (a GABA synthesizing enzyme) (25,36,37), which was not evident in treated patients. Additionally, remission from depression of patients exposed to SSRIs or transcranial magnetic stimulation was shown to be linked with the normalization of GABA levels.

Studies with animal models ($GABA_A$ mutant mice) showed that $GABA_A$ reduced receptor binding leads to an anhedonic phenotype. Several animal models of chronic stress report a decrease of expression of $GABA_A$ receptor in frontal cortex and other brain regions. Another approach to study this NT was through the administration of GABA directly in the hippocampus of rats which protected them from developing learned helplessness (37).

Although some studies have been made in this topic further analysis need to be made in order to ensure robust outcomes.

1.1.5.4 Neurotrophic factors

Neurotrophic factors are key molecules for growth, survival and differentiation of neural cells; in particular neurotrophins are proteins that act in neurons (38)(39).

Brain-derived neurotrophic factor (BDNF) is an important neurotrophin shown to be involved in depression pathophysiology. Clinical studies demonstrated that BDNF levels in the serum were decreased in drug-free MDD patients when compared to healthy participants; other studies reported increased BDNF levels after antidepressant treatment (more prominent in responders rather than non-responders). However, no clear relationship was shown between BDNF levels and depression severity (40).

Animal models of stress also show a downregulation of BDNF in several hippocampal sub-regions (dentate gyrus, CA3 and CA1), which has a negate impact in neuronal plasticity. Also, an experiment where exogenous corticosterone was administrated to rats showed that BDNF expression was reduced in hippocampus. According to that, a single infusion of BDNF into the hippocampus of animals produced a massive and long-lasting antidepressant effect (39). Actually, the majority of the current available antidepressants increase BDNF expression (in regions as hippocampus and prefrontal cortex of animals exposed to stress). Moreover, the efficacy of ADs is very reduced when BDNF expression or TrkB signalling are disrupted (41).

Pre-clinical studies show that an impaired BDNF expression does not lead to depressive-like behaviour but does affect ADs efficacy, apparently it seems that most of the BDNF role is involved with therapeutic action (41).

Not in line with the beneficial effects of BDNF is the finding that inflammation in the brain and some neurotoxins increase brain BDNF levels. In the same way, blockade of BDNF activity in the Ventral Tegmental Area - Nucleus Accumbens (VTA-NAc) pathway exerts an antidepressant-

like effect in rodent models of stress(42). Other findings show that chronic stress cause an upregulation of BDNF in the basolateral amygdala(43).

It is clear that BDNF actions are not always beneficial, so a region-specific BDNF signalling is being studied as well as its impact through epigenetic modifications (e.g maternal separation early in life is capable of influencing this mechanism)(25,42). Epigenetic processes are very dynamic and tissue specific. Some groups have reported a hypermethylation of the *BDNF* gene promoter in *MDD* patients, a post-mortem analysis of patients that commit suicide have reported a lower BDNF expression, in Wernicke's area, associated with an increase of DNA methylation of four CpG sites located at BDNF promotor 4.

Studies are not limited to BDNF, for example **nerve growth factor** (NGF) has also been shown to be decreased in the hippocampus of suicide victims as well as in animals exposed to stress(44). Additionally, NT-3 was also reduced in the hippocampus after stress exposure(45). Moreover, an infusion of this neurotrophic factor in the hippocampus produced an antidepressant response in the forced swimming test (FST)(44).

Another neurotrophic factor implicated in depression is the **glial cell-line derived neurotrophic factor** (GDNF). Some studies showed a decreased expression of this protein in the peripheral blood of depressed patients and reduced levels in the hippocampus were observed in a rodent model of chronic unpredictable stress(46).

1.1.5.5.Neural plasticity

Neural plasticity is a process that include re-organization of dendrites and synapses (synaptic alterations, re-orientation of dendrites and axons and modifications in branching structure) as well as the generation of new neuronal and glial cells, a process called neurogenesis and gliogenesis, respectively. Additionally, long-term potentiation and long term depression are known as functional neuroplastic changes. These physiological neuroplastic changes occur as a response to environmental stimuli, yielding functional alterations and gene expression alterations in order to achieve adaptation and further homeostasis(47,48).

Neurogenesis is the process by which neural progenitors divide mitotically to generate new neurons. This neuroplastic process happens at least in two regions of the adult mammalian brain: the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus(49) and the subventricular zone (SVZ) lining the lateral ventricles(50).

Particularly, the SGZ contains radial glial-like stem cells that express several markers, including GFAP (fibrillary acidic protein) and the intermediate filament nestin. These cells divide asymmetrically and produce Type-2 daughter cells, called transient amplifying progenitor cells or fast proliferating cells, which also express nestin and are much more proliferative than type-1

cells. Type-2 cells give raise to neuroblasts – type 3 cells. Type-3 cells are negative to GFAP and nestin but positive to doublecortin (DCX). This last stage correspond to transition between a slow proliferation neuroblast (which is exiting the cell cycle) to a postmitotic immature neuron that will migrate into the granule cell layer. These new cells will then became maturate into granule neurons with their axons being growth toward CA3 area of the hippocampus. After 2/3 weeks the cells express calbindin a marker of mature granule cells and after 4-8 weeks they are fully integrated in the pre-existing neuronal network(45,51,52).

Several neuroplastic changes have been found in human samples. Hippocampal atrophy is a clear feature seen in meta-analyses studies of depressed patients(53). Additionally, postmortem studies of depressed patients showed reduced glial cell density in the prefrontal cortex, hippocampal DG and anterior cingulate cortex(46,54,55) as well as a decrease in neuronal size observed in the dorsal PFC and anterior cingulate cortex(55,56).

Insights from animal models of depression have produced relevant findings regarding neural plastic changes induced by depression. Dendritic atrophy and spine loss was observed in neurons from the PFC and hippocampus of animal models of chronic stress (57). Also, a reduction in hippocampal neurogenesis was reported in animal models of stress exposure(58). Indeed, hippocampal neurogenesis was shown to be an important contributor for a sustained remission from depressive-like behaviour(59). The relevance of neurogenesis for depression is reinforced by the fact that antidepressant drugs take 3 to 4 weeks to exert their beneficial effects in patients, which corresponds to the same period for the maturation of adult born neurogenesis(45). Not only neuronal cells are affected in animal models of stress exposure. Indeed, chronic stress is known to induce a decrease in the proliferation of glial progenitor cells and reduce the number of GFAP-positive cells in the PFC and hippocampal DG. Additionally, impairments in astroglial cell morphology, metabolism and function are also observed(22,57,60,61).

In fact, astrocytes have an active control on neuronal activity and synaptic plasticity ; this bidirectional communication between neurons and astrocytes is called 'tripartite synapse'. In this process astrocytes are responsible for glutamate clearance from the synaptic cleft through transporters (EAAT1 and EAAT2); conversion of glutamate into glutamine (precursor of glutamate and GABA); release of trophic factors; metabolic support; intervention in the neuronal activity through variations of intracellular $Ca^{2+,}$ among other functions(33,62).

Changes in the number or astroglial cells remodelling may affect the glutamatergic tripartite synapse through a decrease of extracellular glutamate clearance and subsequent activation of extrasynaptic glutamatergic receptors that will result in excitoxicity(22).

To conclude, it was proved that both neuronal and glial neuroplastic alterations, could be reversible after ADs treatment(46,61,63). Treatment with SSRIs, MAOIs or TCA allows a fast

recovery of spine density as well as dendritic neuronal architecture which is associated with the remission from depressive-like behaviour(59,63,64). TCA and SSRIs are also capable of restoring neuro- and glio-genesis as well as BDNF levels(65)(51).

Together, these neuroplastic reestablishments, potentiated by antidepressants, are crucial for the long-term remission from depression(66).

1.2 Validation criteria of animal models

Nowadays, searching for better and accurate animal models is an effort of many researchers. In fact, a wide variety of animal models have been used to mimic the human depression. Many of the symptoms of depression (e.g. depressed mood, feelings of worthlessness, suicidal ideation) cannot be easily measured in laboratory animals because they involve higher cognitive abilities. However, by trying to improve the quality and the validity of the models it is possible to step-by-step get closer to the human disease in order to help finding new therapeutic targets as well as new insights about the pathology seen in the clinics(67,68). Due to this reason, animal models are fundamental and must be continuously improved. For that, several validation criteria must be fulfilled, aiming an intra- and inter-laboratory reproducibility - linked to the standardization power of the model.

In 1969, William McKinney was the first to establish the minimal requirements for animal models of depression. These criteria include: an analogy between the symptomatology of human depression and what is observed in the animals; assessment of clear and objective behavioural changes that should agree between independent observers; treatment effective modalities in humans should reverse the changes observed in the animals; and the existence of reproducibility between investigators(69).

In 1984, Paul Willner refined the validation criteria proposed by McKinney. Most of the researches, working in the field of animal models of depression, rely on this last proposal by Willner that suggests 3 main validation criteria: predictive, face and construct validity. He described predictive validity as the capacity of the model to identify antidepressant treatments through the coherence between animal and human (un)successful agents as well as the model correlation with the success level seen in the clinics(70). Face validity is the resemblance between phenomenological similarities between the model and the disease through a coexistence of several aspects that are specific to depression. In other words, this criterion describes if the model mimics the diagnostic criteria of depression (core symptoms), while not referring to etiological or biological basis(68,70). Construct validity is the capacity to resemble the features and behavioural changes seen in human depression with a solid theoretical rational with unambiguous and homologous interpretation.

Later, Willner updated these criteria for a concept that aligns the theoretical explanation of the human disease with the behaviour and biological dysfunctions seen in the animal model. Etiology is now included in this concept through the identification of the triggering factors that cause a depressive-like state, its characterization (unpredictability, chronic, etc) and its

correlation with biological processes involved in depression(12,68). Willner compared several animal models and stated that predictive validity seems to be the easiest criterion to achieve. Face validity and construct validity are the hardest criteria to get because their validation is filled in only a reduced number of models(70).

More recently, in 2011, Belzung and Lemoine made a reformulation of these classic validation criteria. First, they referred the importance of internal validity which concerns the consistency of the experimental design (reproducibility, inter-observer reliability, randomization, blind experimentation and others) as well as the external validity that refers to the applicability of the results of a study on a sample to its extrapolation to the target population (68). Regarding the validation criteria, they introduced a different perspective of the validity concepts. However, the global outcomes being assessed remained the same, with an exception to homological validity, which is a totally new validity criteria. This criterion refers to the adequate choice of the animal species and of a particular strain. For instance, species more prone to display depressive-like states at behavioural and biological levels are a better choice as an animal model than resilient species(68).

1.3 Animal models of depression~

1.3.1 Animal models based on (bio)chemical manipulations

1.3.1.1 Chronic GC administration

As the name implies, this animal model is developed by the chronic administration of GCs (known to be elevated in depression). These animals display some behavioural alterations similar to those observed in the clinics and also some molecular alterations including: anhedonia and learned helplessness phenotype(12); molecular and cellular changes are observed, like decreased BDNF expression in hippocampus(44), reduced neurogenesis, retraction of dendrites in CA3 pyramidal neurons(71), increased CRF synthesis and secretion(12). These effects are reversed by chronic administration of antidepressants, like amitriptyline (a tricyclic antidepressant)(72).

1.3.1.2 Stimulation of the immune system

Up to 50% of patients with autoimmune diseases, such as multiple sclerosis, experience clinically significant depression. As it was previously stated, the activation of the inflammatory system seems to be correlated with depression onset. Moreover, cytokine alterations seem to elicit central monoamine and CRH changes(72). Induction of endotoxins and cytokines are models that show alterations at different levels, including brain neurochemistry changes, neuroendocrine and neuroimmune function alterations and behavioural changes – coincident with MDD alterations in humans. Additionally, the exposure to endotoxins or cytokines induce anhedonia phenotype, increase stress hormones and decrease locomotor activity and body weight. This is a low cost and easy-to-implement model but with a poor etiological validity.

1.3.2 Genetic-related models

1.3.2.1 Selective breeding

As stated before, depression requires genetic and/or environmental vulnerability. Selective breeding is based on genetic individual differences found in animals, an example is the Wistar-Kyoto (WKY) rat strain(73). This tool can be very useful however more work is needed to establish a line for a reliable model(74).

1.3.2.2 Targeted overexpression or KO (knock-out) of specific candidate genes

These are not considered formal animal models of depression but are helpful in same cases. For instance, 5-HT transporter knockout is used because it is the target of many antidepressants and as such it can give some insights about the molecular mechanisms. Also, the

knockout of tackykinin (NK1) has been associated with stress and anxiety, showing worse performances in the FST.

HPA transgenic animals are also used in this context. These genetic mutant animals express irregular levels of GR, disturbing the normal negative feedback of the HPA axis.(67).

Moreover, it is possible to test candidate-driven mutations, for example to alter a protein known to be implicated in depression and then characterize the resulting phenotype. This reverse genetics allows to start with a gene and study the backwards to identify its function (74).

1.3.3 Lesion model

Bilateral olfactory bulbectomy is a model of depression where the two lobes are ablated. This lesion leads to anosmia and most importantly to the loss of detection of pheromones which are crucial to reproductive behaviour, gender recognition, social dominance among other behavioural and physiological status of the animal(75).

The olfactory system is part of the rat limbic region, and consequently this lesion will lead to a dysfunction of the cortical-hippocampal-amygdala circuit. More so, this model shows changes in behaviour, like impaired food-motivated behaviour, alterations in cognitive tasks (like morris water maze performance) and increased exploratory behaviour/open field activity. Also increased activity of the HPA axis is observed and some evidences claim that there are alterations of the immune system response.

Importantly, this model shows predictive validity because chronic administration (not acute) of antidepressants can revert behavioural, endocrine, immune and neurotransmitter changes(75). However, this model lacks etiological validity because, in humans, the loss of olfaction does not produce self-rated depression(68).

1.3.4 Animal models based on stress exposure

Depression is a disease with a highly influence from environmental factors(16). Actually, models based on environmental stressors have great aetiological validity compared to the previously mentioned (brain lesions or biochemical manipulations)(12).

1.3.4.1 Acute stress models

These models are used as tools to rapidly screen putative antidepressant compounds. An example is the **FST** that consists of placing the animal in an inescapable cylinder tank filled with water. The animal starts struggling, swimming and climbing and eventually will stay immobile. It is measured the amount of time that the animal takes to stay immobile. One strength of the

model is the reduced errors in results measurement, however it lacks some validation criteria since the phenotype is reversed by acute administration of antidepressants(12,72).

Still within the learned helplessness category, another approach is to expose the animals to **inescapable electric shocks** that allow them to develop a "helplessness" state. This happens because when animals are re-exposed to shocks, with an easy way out, they display a big latency or fail completely to escape. The major weakness is the same as above, successful response to acute drug treatment. Regarding positive aspects of the model, it is relevant to mention that rats develop alterations in sleep patterns; alterations in HPA axis activity; decreased number of synapses in the hippocampus; elevated CRF and corticosterone levels which all correlate with the human disease (74)(72).

1.3.4.2 Chronic stress models

Psychosocial stress (defeat or social isolation)

This is a model with an interesting etiological validity. In social defeat the animal is repeatedly exposed to a dominant/aggressive animal. These animals show a reduced preference for sucrose in the sucrose preference test (SPT) and changes in the neuroendocrine system, which are reversed through chronic administration of antidepressants. Despite the fulfilment of the validation criteria, social behaviour is not well characterized in rodents. Also these models have poor reproducibility which reduced their application(72).

Maternal care (maternal separation or prenatal stress)

This is an early life stress model that produce neuroendocrine and behavioural changes. For example, it is seen that a bigger vulnerability to learned helplessness, persistent into adulthood, and hyperactive HPA axis. Their reproducibility is relatively good and successful results are shown in many different species from rodents to non-human primates. Antidepressants administration can reverse these abnormalities(72).

Chronic stress exposure in adulthood

These models are among the most valid ones(70). A crucial explanation for that is their naturalistic essence(74). As mentioned before, stress is one of the most consensual precipitating factors for depression.

There are two widely used models of chronic stress: unpredictable chronic mild stress (uCMS) and chronic unpredictable stress (CUS)(76,77). The first one refers to a permanent exposure during 6 weeks to mild stressors in an unpredictable manner(77), whereas CUS protocol

is an intermittent exposure (1h per day) of more aggressive stressors during a 4 week period (76). Chronic unpredictable stress models reveal increases in plasma corticosterone levels, augmented serotonergic activity in the hypothalamus (76), alterations in aggressiveness, sexual behaviour as well as grooming deficits. These phenotypes are reversed by chronic administration of antidepressants either during the stress or as a post-stress treatment(72).

Katz and colleagues were the first to develop a chronic mild stress procedure(78). Later, Willner developed a protocol based on this previous one but reduced the severity of the stressors to increase the resemblance with the human daily life stressors. Moreover, he considered reactivity towards a reward as the most relevant behavioural test to assess the phenotype onset, instead of locomotor activity previously used by Katz(79). This Willner procedure consists of a permanent exposure to a variety of mild stressors (e.g. overnight illumination; periods of food and/or water deprivation; cage tilt; change of cage mate, damp bedding), which must be changed every few hours(77) during a period of several weeks(80). The diversity, unpredictability and mild intensity of the stressors are crucial aspects to prevent habituation to the protocol.

Many applications are possible through the uCMS, this protocol can be used to discover new antidepressant drugs but also to provide insights about the pathophysiology of the disease (in both, cellular or molecular scale)(80).

A closer look at uCMS model allow us to consider it as one of the most valid for the 3 main criteria (predictive, face, construct and homological)(68,77), as it is explained below.

A fulfilment of the predictive validity is observed because both behavioural and molecular alterations can be reversed through chronic administration of antidepressants from different classes (MAOs, SSRIs, TCAs)(5,64).

Regarding face validity, it is possible to observe a decreased preference for a palatable sucrose solution(77,81), a diminished sexual behaviour, locomotor activity as well as alterations in sleep changes and self-care(77,79). Not only mood is impaired, cognition and anxiety are also affected in this model, resembling the multidimensional nature of depression in humans. Stressed animals show high anxiety levels and a worse performance in cognitive tests when compared to control animals(5). All together, these findings cover the main symptoms observed in humans and can be seen in this animal model.

Construct validity is the theoretical rationale behind the alterations reported. For that, molecular and cellular analyses are needed. Indeed, increased activity in the HPA axis, which includes adrenal hypertrophy and corticosterone hypersecretion are observed in this animal model. Downregulation of hippocampal 5-HT_{1A} receptors and hippocampal GR as well as reductions in frontocortical and hippocampal BDNF protein are also observed(80). Moreover, it is possible to observe abnormalities in the immune system(77), altered neuroplasticity

(neurogenesis is reduced, dendritic length and morphology is impaired in several brain regions affected with depression)(22,66,82).

Lastly, concerning the new criteria - homological validity, it has been reported that the sensitivity of the model varies between strains; however, the effectiveness of the procedure was reported in several different strains(68,79). Rats tend to be better models than mice in chronic mild stress models, and among the most used strains are Wistar Han and Sprague Dawley(79). Specific strains require specific adaptations in the procedures, for example Wistar Han rats only show robust measurements in SPT (anhedonic test) if the sucrose concentration is 2% and not 1%(77).

It is thus consensual that the model is valid in all criteria. However, some weaknesses can be pointed out: it is difficult to implement because it is executed manually what implies a labour-intensive work; also it takes a long duration and it is space demanding(79). Some intrinsic aspects are also less favourable, like the use of artificial physical stressors (strobe lights or tilted cage) and the fact that non-treated animals reverse the phenotype in all the behavioural tests (except SPT) after 10 weeks of the uCMS. Despite several independent research groups had proven the reliability of the model his lack of reproducibility still remains a big concern and is seen as the biggest limitation(79). Some authors have suggested divergences in protocols, handling influence, disparity in conditions from the animal facilities as well as variations in animals. This last feature may account for different genetic backgrounds as well as different microbiota of each animal, known to play a role on susceptibility of animal models of chronic stress(79,83). However, no clear evidences have emerged to explain the variability observed between laboratories. As such, one of the ways to improve the uCMS model is to increase standardization as well as to increase its effectiveness.

1.4 Research Objectives

The main goal of this work was to finish the development of an automated equipment to induce depressive-like behaviour in rodents, based on the uCMS protocol and to do a pilot validation of the equipment.

For that, the following objectives were defined:

- Participate and supervise the construction of the equipment;
- Test and improve the automated stressors individually (in order to better resemble the original manual method);
- Validate the automated protocol using previously determined validation criteria:
    - Determine the induction of molecular, morphological and biochemical alterations in the automated model and compare with the manual protocol (construct validity);
    - Assess changes in several behavioural dimensions in the automated model and compare with the manual protocol (face validity);
    - Explore the pharmacologic ability to reverse the behavioural, molecular and cellular changes in the automated protocol (predictive validity)

2. MATERIALS AND METHODS

The development of the equipment was performed in the context of a multidisciplinary team composed by a professional industrial designer, a mechanical engineer, an electrotechnical and computer engineer as well as neuroscience professors and researchers.

The overall concept and design of the rack was already done when I joined the team, but the construction, optimization and implementation of the system was performed during my master's thesis project with my direct intervention and active collaboration.

Both, the equipment and the modified stressors were optimized and improved, first in empirical experimentations with several materials and architectures and, in later tests, through the use of animals for each individual stressor – from both models of chronic stress, CUS and uCMS.

After this, all stressors from the uCMS protocol were put together to perform a complete protocol with animals. In order to perform the proposed study, two groups of animals were used: one group was submitted to the manual (traditional) protocol (uCMS) and another using the automated equipment (auCMS). A subset of animals from this latter group was submitted to fluoxetine treatment (treated auCMS). Also, a group of animals was kept in the accommodation room and only handled by the experimenters - the control group.

Animals from all the groups were evaluated in order to measure behaviour, neuronal morphology in the hippocampal DG, corticosterone levels and cell proliferation. Details on the animals, treatments and tests are given along chapter 2.

2.1 Animals

2.1.1 Optimization phase

Male Wistar Han (Charles-River Laboratories) *Rattus norvegicus*, with ~1 year of age and weighing 400-500g, were kept 2 or 3 per cage, under 12h light: 12h dark cycles, at 22ºC, relative humidity of 55% and with food and water ad libitum.

Some animals were used to test the adaptions of each stressor, individually: 3 animals for tilted-cage stressor; 1 animal for shaking stressor (additional); 3 animals for water-involving stressors; 6 animals for confinement stressor and also 3 animals for food deprivation followed by exposure to inaccessible food (additional).

2.1.2 Pilot-study

Male Wistar Han (Charles-River Laboratories) *Rattus norvegicus*, with 2 months of age and weighing 300-400 g, were kept 2 or 3 per cage, under 12h light: 12h dark cycles, at 22ºC, relative humidity of 55% and with food and water ad libitum.

Four groups were defined:

A - 10 animals exposed to uCMS (uCMS)

B - 6 animals exposed to auCMS without treatment (auCMS, NT)

C - 6 animals exposed to auCMS with treatment (auCMS, T)

D - 10 control animals (C).

A, B and D were injected with a saline solution, whereas C was treated with an antidepressant (fluoxetine). In Group D, animals were not submitted to any chronic stress protocol.

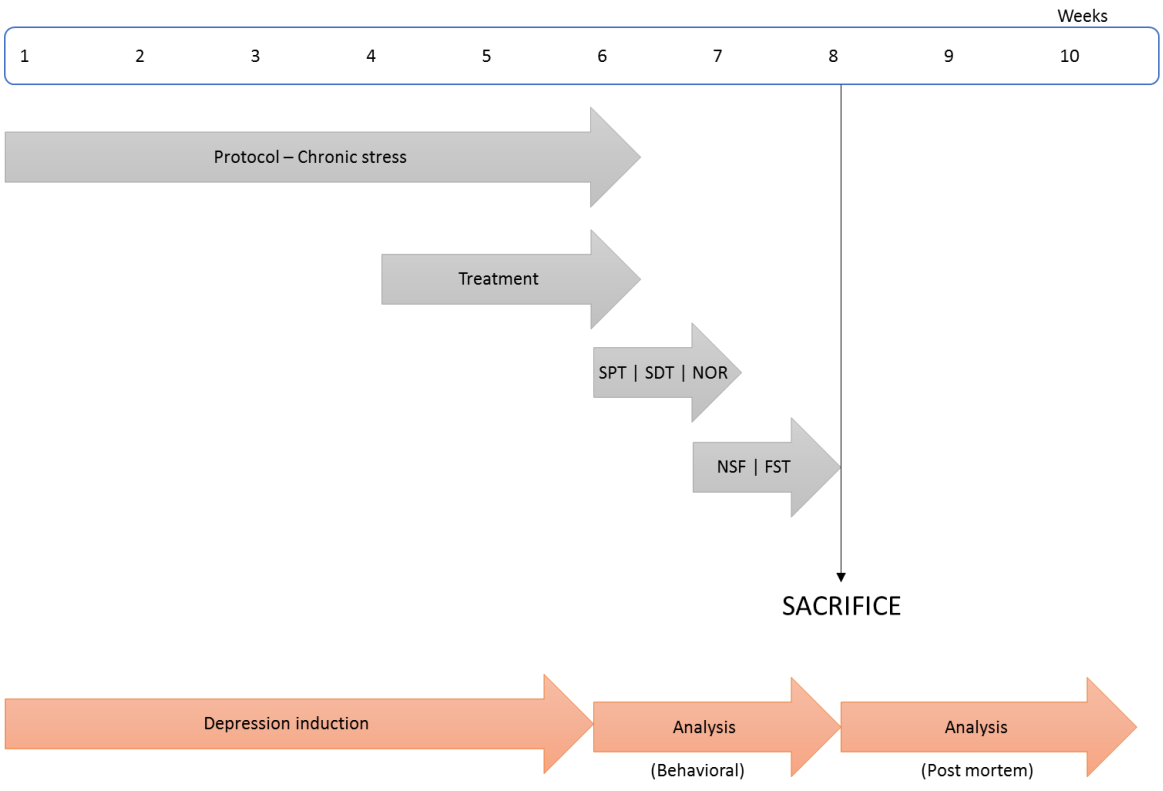

Figure 2. Timeline of the procedures: The induction of chronic stress was performed manually or automatically during 6 weeks and in the last two weeks of this protocol fluoxetine was administered. After, depression induction protocols several analysis were performed.

2.2 Procedures

The construction and optimization of the rack was performed in the lab facility. Only necessary tools were built outside the lab, in the mechanical facilities.

After the construction of the equipment, it was necessary to adapt the individual stressors. For that, tests with animals were performed to achieve the most suitable configuration. The equipment was placed in the lab facility but outside the animal facility. So, tests that involved the use of animals in the equipment were performed outside the animal facilities (tilted-cage adjustments, shaking tests, and stressors performed with water) with the sacrifice of the animals at the end of the experiments. Tests that were not dependent of the equipment, like confinement or measurements of plasma CORT levels of animals, were tested in the animal facility.

This process took around 6 months. The other 6 months were used to do a pre-test of the automated uCMS protocol and its further analysis.

In Fig 2, it is possible to have an overview of the whole sequence of the procedure. Below, there is a detailed description of the induced protocol and the following analysis.

2.2.1 Manual unpredictable chronic mild stress

A modified version of Willner's uCMS protocol was applied(5). The variety range of stressors included confinement to a restricted space, placement in a tilted cage (~30°/45°), housing on damp bedding during the night, overnight illumination, food deprivation followed by exposure to inaccessible food, water deprivation followed by empty bottle exposure, reversed light/dark cycle, exposure to strobe lights, overcrowding, cage switch and startle noise. Animals are randomly and uninterruptedly exposed to the stressors during 6 weeks.

In general, our protocol had 4 stressors each day and 1 stressor in the night. Stressors for the day did not exceed the following time period: restricted space (2h), tilted cage (4h), inaccessible food (1h), empty bottle (1h), strobe lights (4h), overcrowding (3h), cage switch (2h) and startle noise (4h).

The intensity level (e.g the time of exposure/ aggressiveness of the stressor/ double stressors) increased over the development of the protocol(5). Double stressors were used in both night and day period; they included: water deprivation with illumination, food deprivation with tilted cage, tilted cage with reversed light cycle, food deprivation with wet bed and water deprivation with strobe lights.

Control animals were gently handled every week throughout the 6 weeks protocol.

2.2.2 Prototype

The traditional method to perform this protocol is manual. Animals are housed in a rack and every 2 or 4 hours the experimenter needs to change the stressor, with the exception of the night stressors. Through the development of this novel equipment it is possible to apply uCMS, in an automated manner with all the cages simultaneously performing the stressors.

The structure of the equipment was designed based on a traditional rack. This automated rack is connected to a computer where it is possible to upload the scheduled protocols with the intended stressors as well as the lights state. Because the stressors incorporated into the automated prototype are based on the same stressors used in manual protocols, the outcomes may be preserved.

The equipment was designed for 24 cages (3 animals per cage), which enables the manipulation of 72 animals at the same time.

The patenting of this prototype is in process of submission.

2.2.3 Automated unpredictable chronic mild stress

This equipment will enable to perform the same manual stressors of a traditional uCMS protocol but in an automated way. The protocol was uploaded into the computer (connected to the automated rack) and it ran over 6 weeks.

Stressors were divided in 3 categories: totally automated (TA), partially automated (PA) and non-automated (NA), as it is deeply explored in the Chapter 3 (results).

TA stressors are the ones exclusively performed by the rack, without the intervention of the operator and include placement in a tilted cage (45°), housing on damp bedding during the night, overnight illumination, reversed light/dark cycle and startle noise. PA stressors refer to confinement to a restricted space, a stressor that has undergone some changes in order to be user-friendlier. Finally, NA stressors are those that depend exclusively from the intervention of the experimenter and for that were kept as in the manual protocol; that is the case of food and water deprivation followed by exposure to inaccessible food/water, overcrowding and cage switch.

2.3 Drugs administration

Fluoxetine (SSRI) was the antidepressant chosen to test the reversion of the symptoms(84) because it is one of the most widely used first-line therapy in the clinics and it was already shown to be able to induce recovery from uCMS in depressive-like rats(5,59).

The treatment groups received an intraperitoneal injection (10mg/kg, Kemprotec) every day during the last two weeks of the uCMS protocol, ensuring its action during all the behavioural tests period(5). The remaining groups (group A, B and D) received an I.P. injection of saline solution.

2.4 Behavioural Tests

In order to validate the newly developed automated equipment all animals (controls, uCMS-exposed, and auCMS-exposed) were subjected to a behavioural test battery which encompassing tests to assess the three major behavioural domains affected in depression.

Behavioural tests were performed at the end of the uCMS protocol starting on week 6. To evaluate mood improvements three behavioural tests were used, the SPT and the sweet drive test (SDT)(85) to evaluate anhedonia as well as FST(5) to evaluate behavioural despair. In order to analyze changes in cognition we used the novel object recognition (NOR) test (5). Anxiety-like behaviour was evaluated using the novelty supressed feeding (NSF)(5) test.

The tests were performed in the following order SPT-SDT-NOR in the 6th week and NSF-FST in the 7th week as it is represented in **Fig 2**.

2.4.1 Sucrose preference test

Sucrose Preference Test (SPT) was used to assess anhedonic behaviour of the animals exposed to the uCMS protocol(5). Animals were allowed to habituate to the sucrose solution (2% m/v) one week prior to the uCMS protocol in a three-trial paradigm in order to establish the baseline values for sucrose preference. For each assay, animals were food- and water-deprived for 12h during non-active period (light on/diurnal period). The room was cleaned with ethanol 96% and the test was performed under dim illumination. Each animal was placed individually in a cage, covered with the grid and the lid. Two pre-weighted bottles were placed in opposite sides of the cage: the one containing a 2% (m/v) sucrose solution in the food site and the one with tap water placed in its original site.

Sucrose preference was calculated by the following formula: sucrose preference = [(sucrose consumption / Total consumption) x 100]. Anhedonia was defined as a reduction in sucrose preference in relation to the baseline levels(5).

2.4.2 Sweet drive test

SDT was also used as a measure of anhedonic behaviour, as previously described (85). Animals were pre-habituated to sweet pellets (3.77 kcal/g; Honey Cheerios®; Nestlé Portugal S.A.,

Portugal) in the day before the first trial, overnight. Before each trial, animals were food-deprived for 12h during the light (non-active period) and the exposure to stressors was suspended. The test apparatus consisted of a black acrylic enclosed arena (82 cm x 44 cm x 30 cm) divided by transparent and perforated walls into 3 closed chambers and one pre-chamber in which the animal is initially placed. This pre-chamber is connected to a middle chamber by a trap door.

Once the animal crossed the trap door, this door closes and the animal is allowed to explore de other 2 chambers, one on the left and one on the right side of the apparatus. Part of the apparatus was also a transparent acrylic lid to ensure surroundings noise-reduction.

When initiating a new trial, the SDT arena was carefully cleaned with ethanol 10%. A total of 20 pellets of regular food (3.60 kcal/g; Certificate standard diet 4RF21; Mucedola, S.R.L., Italy) were positioned in the corner of the left chamber and 20 sweet pellets were placed in the corner of the right chamber. The animals were allowed to explore freely for 10 minutes (min) per trial.

At the end of the trial, pellets consumption was determined and preference for sweet pellets was determined as follows: preference for sweet pellets (%) = Consumption of Sweet Pellets (g) / Total Food Consumption (g) × 100.

2.4.3. Novel object recognition

Cognitive function was assessed through the NOR at the 6th week of the uCMS protocol. For this purpose, a black acrylic box (50x50x150cm) with an open field space (51x51x39.5cm) and illuminated with a white lamp (100-140 lux) was used (86–89).

The test was divided in 4 days. On the first day the animals were allowed to explore the test apparatus without any object inside, during 10 minutes. On the second day, two identical objects were placed in the back left and right corners of the apparatus and the animal was able to explore them during 10 min. On the third day, the object in the left corner was replaced by a novel object for 3 min in order to check long-term memory. Lastly, on the fourth day, animals were tested for short-term memory. The previous objects were changed for two novel equal objects and the animal was allowed to explore for 10 min. Within an interval of approximately one hour, the left object was replaced by a new object and the animal was allowed to explore once again, for 3 min.

Trials were video-recorded and analyzed in the Etholog (vs.2.2) software. The discrimination index (D) was calculated by the following formula: D = (N-F)/(N+F); being N the time spent exploring the Novel object and F the time spent exploring the Familiar object.

The exploration of an object was considered when the animal is directing the nose to the object at a distance of less than 2 cm or touching it with the nose.

2.4.4 Novelty supressed feeding

NSF was used to measure anxiety-like behaviour at the 7th week of the uCMS protocol. For this test, animals were food deprived for 18h and then placed for 10 min in an open-field arena covered with bedding, containing a single food pellet at the centre. When reaching the pellet, the animal was immediately placed individually in the home cage and allowed to feed a pre-weighted regular food pellet for 10 min.

The latency to feed in the open-field arena was used as an anxiety-like behaviour index(5,85).

2.4.5 Forced Swimming Test

Learned-helplessness was evaluated through the FST (85) and was conducted at week 7 of the uCMS protocol. In the test, animals were placed in transparent glass cylinders filled with water (25°C; 50cm of depth) and submitted to a 5 min pre-test session, 24h before the assay. The test session had a duration of 5 minutes.

Trials were video-recorded and immobility time was measured through the Etholog (vs.2.2) software. Learned-helplessness was defined as an increase in the immobility time.

2.5 Plasma corticosterone level

The blood samples were collected from all animals, in the 6th week (at 8 p.m and 8 a.m), kept on ice and then centrifuged immediately for 15 min. The obtained plasma was kept at –80 °C until analysis.

Corticosterone levels were measured using a commercially available kit (Corticosterone ELISA kit –ABCAM, ab108821).

2.6 BrdU Immunostaining

In the last day of the protocol, animals were given a single intraperitoneal injection of Bromodeoxyuridine (10mg/kg, Sigma-Aldrich). BrdU is a thymidine analogue that incorporates into DNA during the S-phase of the cell cycle.

Twenty-four hours after the injection, animals were deeply anaesthetized with sodium pentobarbital (Eutasil, Ceva Saúde Animal) and transcardially perfused with saline solution followed by ice cold 4% paraformaldehyde (PFA, ThermoScientific). After, brains were removed and post-fixed in 4% PFA. Twenty-four hours later, brains were washed with phosphate-buffered

saline (PBS) solution and transferred into a 30% sucrose solution until they sank. Then, they were embedded in OCT compound (Thermo Scientific) and frozen using liquid nitrogen and 2-methyl-butane. Finally, brains were frozen at -20ºC until being cut in a cryostat.

Coronal sections (20µm) were cut in a cryostate, extending over the entire length of the hippocampus and after stained for BrdU (1:100; anti-rat, Dako)(52).

Briefly, tissue sections were fixed with PFA 4% and washed with distilled water. Antigen-retrieval was performed with a citrate solution for 20 min in the microwave. Next, sections were washed with distilled water. Permeabilization was made with PBS-Triton for 10 min followed by acidification with HCl for 30 min. Then, the slices were washed with PBS-Triton, and were after incubated overnight with the primary antibody. Finally, a secondary antibodies incubation for 2h (1:1000; anti-rabbit Alexa-fluor® 494; Life Technologies, Thermo Fisher Scientific) was performed followed by slides washing with PBS-Triton. At the end, all sections were stained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen; 1:200 in PBS-Triton) for 10 min.

For each animal, BrdU positive cells within the SGZ of the DG were counted (3-8 brain sections per animal) using a fluorescence microscope (BX61, Olympus, Hamburg, Germany). The corresponding DG areas were determined using a stereologic microscope (Bx51, Olympus, Tokyo, Japan)(52).

2.7 Morphological analysis

For the three-dimensional morphometric analysis, animals were deeply anaesthetized with pentobarbital (Eutasil, Ceva Saúde Animal) and transcardially perfused with 0.9% saline. Then, brains were removed and immersed in Golgi-Cox solution for 21 days; transferred to a 30% sucrose solution and cut on a vibratome. Coronal sections (200µm) were collected in a 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak), fixed in Kodak Rapid Fix, dehydrated and xylene cleared before coverslipping.

Dendritic arborization was analyzed in the dorsal dentate gyrus. For each selected neuron, all branches of the dendritic tree were reconstructed at 1000x (oil) magnification using a stereologic microscope (Zeiss) and Neurolucida software. For each animal, a minimum of 8 neurons were studied and total dendritic length was determined (64). Three-dimensional Sholl analysis was used to evaluate the arrangement of the dendritic material; for this, the number of dendritic intersections with concentric spheres positioned at radial intervals of 20 µm was determined(63).

2.8 Statistical analysis

Statistical analyses was performed using GraphPad Prism 6 software (GraphPad Software, Inc.)

One–Way analysis of variance (ANOVA) was used to compare between CT group (D), auCMS untreated group (B) and uCMS group (A). If H0 was rejected, differences between groups were determined by Tukey's multiple comparison test.

Student's t-test was used to test differences between auCMS treated (C) and non-treated (B) animals.

Two-way analysis of variance (ANOVA) was used to assess differences between CT group (D), auCMS untreated group (B) and uCMS group (A) specifically for CORT levels and sholl analysis (morphology). If H0 was rejected, differences between groups were determined by Sidak's multiple comparison test.

Statistical significance was accepted for $P \leq 0.05$. Data is presented as group mean ± standard error of the mean (SEM).

3. RESULTS

3.1 Rack development and optimization

In this section I will start by presenting the steps in which I was involved during the construction phase of the prototype. Then, I will approach the optimization of the mechanisms and issues that were raised during the implementation of the stressors. To conclude, I will focus on the behavioural, cellular and molecular analysis that result from the pilot study using a small cohort of rats.

3.1.1 General features

This equipment (**Fig 3**) presents several features that allow overcoming some of the weaknesses of the uCMS protocol. Moreover, the equipment is controlled and programmed through a computer present in the same room (**Photo 1**).

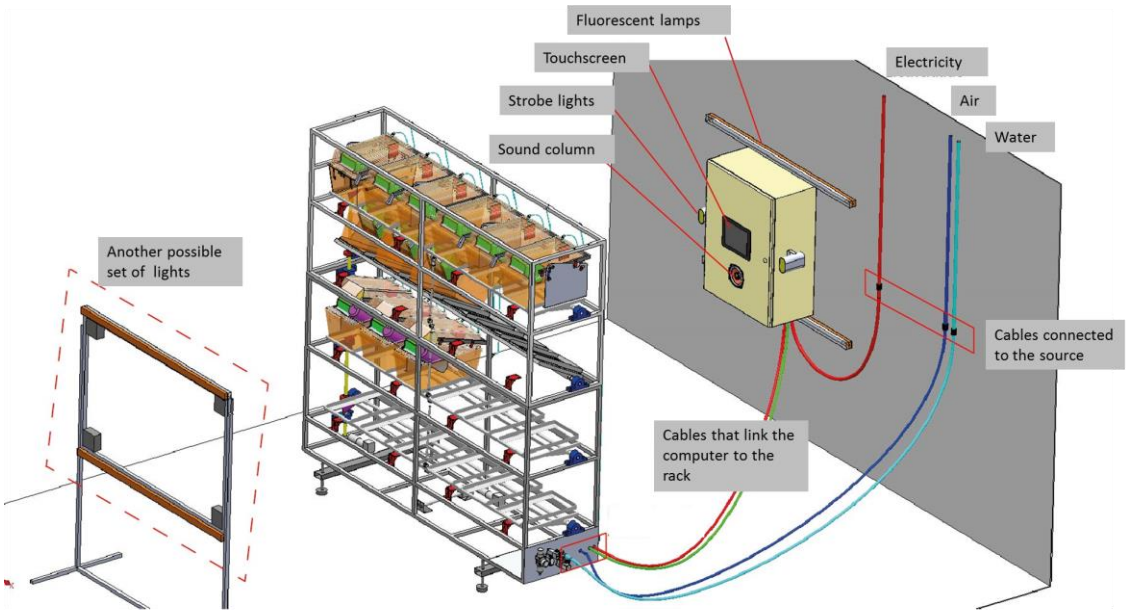

Figure 3. Representative scheme of the automated prototype for uCMS.

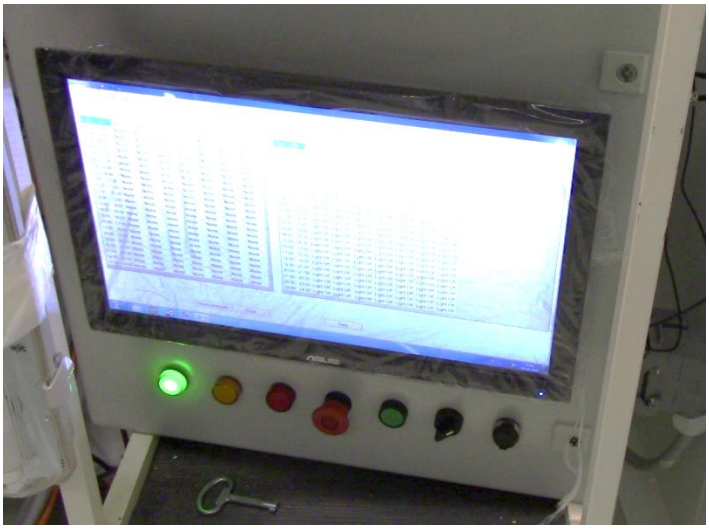

Photo 1. Control computer linked to the equipment.

3.1.2 Mechanical details

This is a labour intensive protocol, in particular the manipulation of the cages during some of the stressors. To overcome this issue a metallic structure bounded to the rack and to the lid of the cages, with the aim of opening 3 cage lids at once, was developed.

This feature (Photo 2) is a user-friendly structure very useful to stressors that involve manipulation of the cages by the experimenter, particularly to perform stressors of deprivation/inaccessibility of food or water as well as to perform our proposed confinement stressor. Also, it is helpful for stressors dependent from animals' manipulation, like overcrowding or switch-cage.

Photo 2. Mechanical feature of the equipment – opening three cages at once.

3.1.3 Stressors categories

Regarding the stressors, they were divided in 3 categories: totally automated (TA), partially automated (PA) and non-automated (NA), as mentioned in the Chapter 2 (methods).

Below, there is a list of the stressors that are part of the uCMS protocol, characterized by their categories:

Table 1. Stressors of the model divided by 3 main categories.

TA	A.	Tilted cage (approximately 45º)
	B.	Housing on damp bedding during the night
	C.	Overnight illumination
	D.	Inverted light/dark cycle
	E.	Exposure to strobe lights
	F.	Startle noise
NA	G.	Food deprivation followed by exposure to inaccessible food
	H.	Water deprivation followed by exposure to an empty bottle
	I.	Overcrowding
	J.	Cage switch
PA	K.	Confinement to a restricted space

3.1.4 Totally Automated stressors

Totally automated stressors are performed exclusively by the rack without the experimenter intervention. This category encompasses motor-dependent stressors (A), water/air supply-dependent stressors (B), lights-dependent stressors (C, D, E) and sound-dependent stressor (F).

Tilted-cage (A)

In the original protocol the cages are placed on the floor or in a table with a grid below them to enable their inclination.

Our automated equipment has a motor incorporated to allow the implementation of some stressors which is the case of tilted-cage (**Photo 3**). The choice of the angles must be previously programmed, meaning that different angles cannot be incorporated into the same protocol.

Photo 3. Mechanical feature of the equipment – tilted cage stressor

Shaking (additional stressor - CUS protocol)

This motorized system was also developed to perform shaking, which is a stressor from the CUS protocol. Although this stressor is not part of the uCMS protocol (it was not included in the set of experiments of the pilot study described in this work), it was introduced to extend the features range for future work.

During the tests made to refine the equipment, it was observed that shaking speed was not uniform for all the rack lines. After analysing several causes, we realised that the speed variations were due to a mechanical imperfection (the cam did not rotate closest to the Teflon board) and as such, the problem was solved by putting a metallic ring (**Photo 4**) near to the spring's system.

Photo 4. Mechanical detail – metallic ring for the shaking movement correction

Damp bedding (B)

In the manual protocol, water is added to the cages one by one. On the other hand, this automated rack enables to provide a uniform and simultaneous water supply to each cage through a tube system linked to the cage lids (**Photo 5**). The quantity of water released can be

programmed. In order to get this water/air supply system operational an extensive process of optimization was made.

First, we had to find the most suitable diameter for the tubes, capable of supplying water until the most high and distant corner of the rack as well as the optimal material for the tubes' system. After a few empirical experiments we observed that 16mm was the minimum tube diameter to achieve an equal water distribution. Regarding the materials' choice, a low-density polyethylene tube was selected, based on a drop wise irrigation. This material is not toxic or harmful to the animals and does not erode with water. Nevertheless, other liquids should not be used inside the tubes.

To prevent the junctions of the tubes of pouring water, some clamps (**Photo 6**) were used for waterproofing the system

Additionally, the system has also the particularity to supply air (using the same system of tubes used for water). Experiments were made to find the most suitable air pressure to resemble hot air stream in the CUS protocol. We concluded that each cage needs to have at least 6 bars of pressure to perturb the animals similarly to the manual stressor. Although, at first this air system was built as an independent stressor, to mimic hot air stream, after some experiments this aim was abandoned. For now, the air will only be used to purge the system after stressors involving water. The objective is to dry the tubes after each use to prevent the growth of fungi and bacteria.

Photo 5. Water/air supply system – wet bed or cold water stressor

Photo 6. Detail of the water tubs – clamps to seal the system

Regarding the architecture of the structure, some attempts were made and a final structure was found. An approach that divides the structure in two (1st, 2nd line and 3rd, 4th line) appeared to be the one that minimizes the water volume error for each cage. Even though, to equalize the volume of water per cage some mini taps were introduced near the tube exit to regulate the water flow per cage (**Photo 7**).

It must be highlighted that, in the beginning of each protocol, the system needs to be pre-tested and mini-taps must be adjusted, in fact the pipes need to be filled with water to achieve equal volumes of water. For that, before each protocol a priming process must be performed to fill the tubes, otherwise the pre-defined times will not lead to a standard volume.

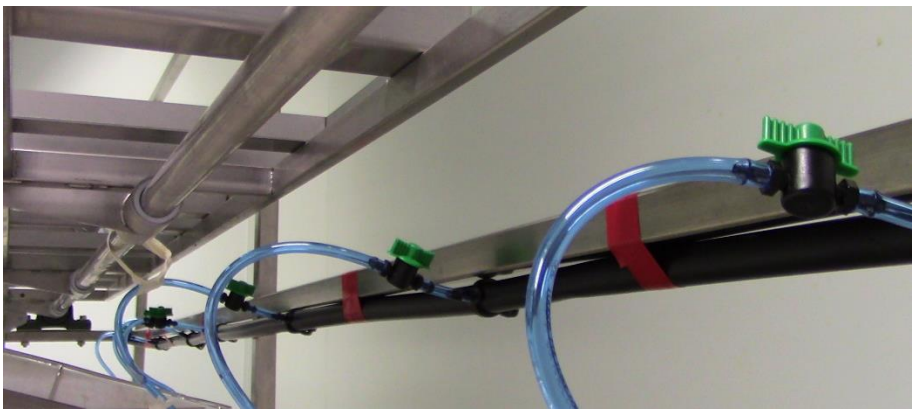

Photo 7. Detail of the water tubs – mini taps to control water volume

Cold water (additional stressor - CUS protocol)

Our system provides the chance to perform another stressor from the CUS protocol - cold water exposure. In this stressor, animals have their paws immersed in cold water in a cage without bedding. This stressor was not included in the pilot validation work. However it just require a

definition of the exact quantity of water to perform this stressor and its programming in the system.

Light-dependent stressors

Several stressors (C, D, E) are light-depend. Lights and stressors settings must be programmed as separate elements in the software, since most of the times they are used in parallel with other stressors; for example inverted light-cycle with tilted cage. (Photo 8). For the E stressor is was necessary to incorporated strobe lights in the system (Photo 9).

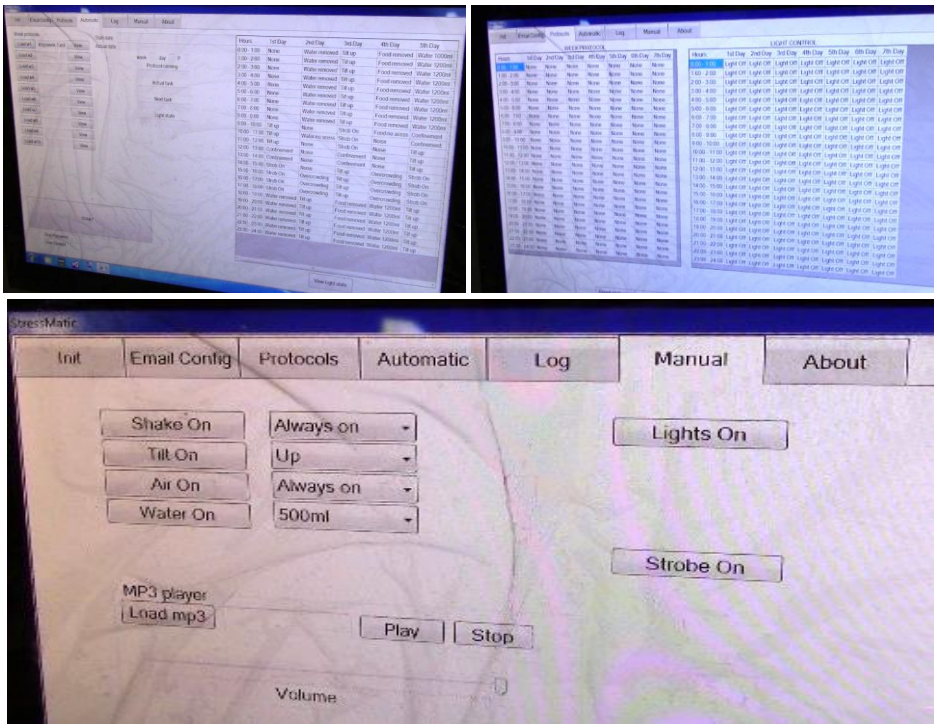

Photo 8. Software that controls the equipment

Photo 9. Accessories linked to the equipment – strobe lights stressor

Sound-dependent stressor

Lastly, to perform the startle-noise stressor (F) a sound-system was linked to the rack and put under the software's control (**Photo 10**).

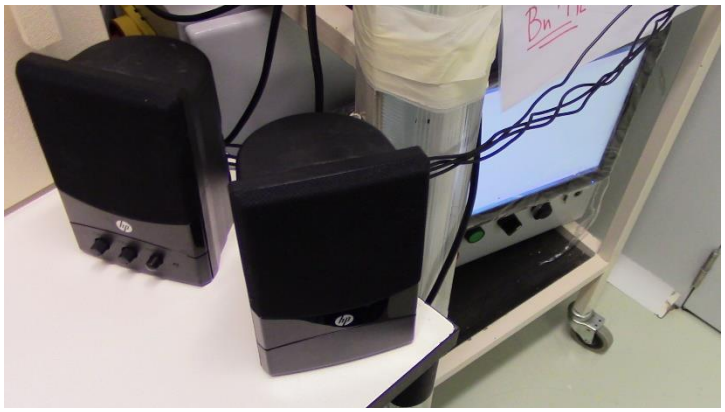

Photo 10. Accessories linked to the equipment – startle noise stressor

3.1.5 Partially automated stressors

Confinement to a restrict space

Partially automated stressors are not performed by the rack but were modified comparing to the manual protocol to reduce the intervention of the experimenter.

In the manual protocol, confinement is performed by putting 3 animals in a plastic box; in this alternative version of the protocol, an acrylic object was designed to reduce the space of the cages and confine the animals (**Fig 4**).

In the development of this stressor, it was necessary to measure the volume of the plastic boxes used in the manual protocol (3L). Our device was designed and built to try to produce the same effect of the manual stressor.

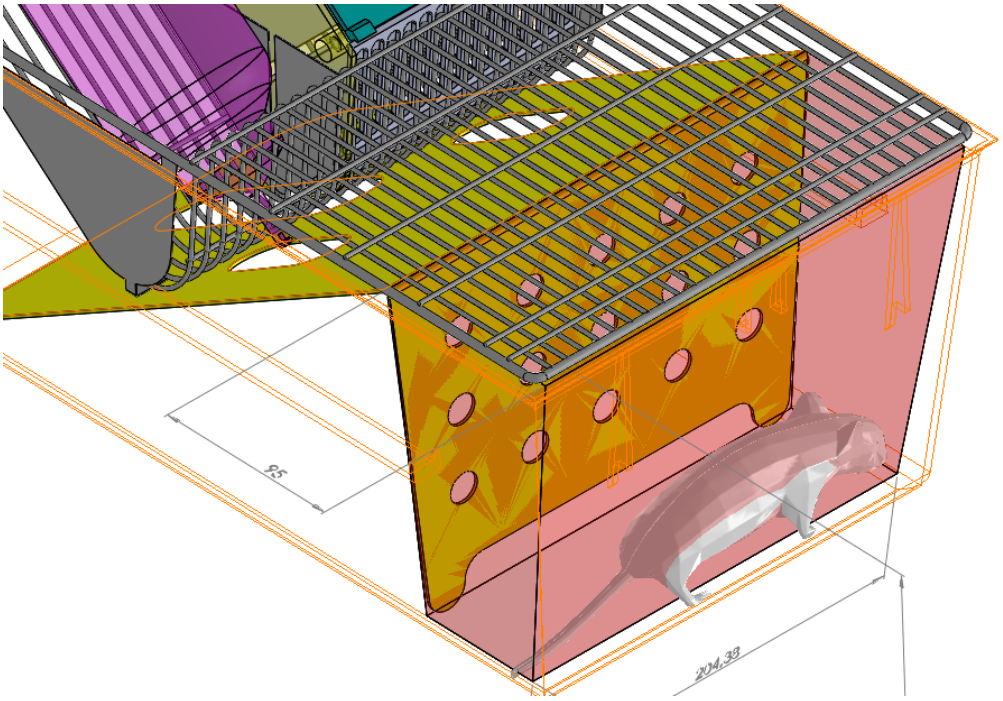

Figure 4. Demonstration of the operation mode of our space restrictor.

Food deprivation followed by exposure to inaccessible food (additional stressor)

In the original protocol, to perform this stressor the food is removed from the cages by the experimenter at the end of the day and in the morning after a container is placed in the grid to allow the animals to smell but not to eat the food (during a period of 1 hour).

To try to reduce this work, an accessory was constructed to replace the food deprivation stressor (G). A food container was built to allow an easy access or restriction of the food just by changing its position.

The different versions of this accessory will be further explained (**Photo 12**).

3.1.6 Non-automated stressors

Non-automated stressors are not performed by the equipment; instead they are completely dependent on the experimenter's intervention. These stressors are G, H, I and J, which are described in **Table 1** and were not modified compared to the traditional protocol.

3.2 Optimization of individual stressors with animals

At this point, stressors were already developed. Since some of them suffered several alterations, it was necessary to guarantee that the effect provoked in the animals was similar to what happens in the manual protocol (e.g. hypercortisolemia). For that purpose modified stressors were tested individually with rats.

3.2.1 Total Automated stressors

Tilted-cage (A)

In the manual protocol tilted cage is performed with around 30°. Because our prototype gives us the possibility of having an inclination of more than 60 °(5), it was necessary to test with the animals different inclinations to find the most proper one, more precisely, the inclination that induces discomfort to the animals. We chose ~45 °, a bit higher than what is used in the manual protocol. This inclination increase was made to improve the efficacy of the procedure (since this stressor was really mild) without compromising it.

Shaking (additional stressor - CUS protocol)

After adjusting the shaking speed a first trial with animals was performed. The stressor was too mild to disturb the rats and the movement needed to be more violent to mimic the manual stressor. For that, we decided to build a bigger cogwheel to achieve the required speed (this solution was chosen instead of changing the cams because it would take a more mechanical expertise and it would be more expensive).

Damp bedding (B) and cold water (additional stressor - CUS protocol)

A test with the animals was made to find the quantity of water needed for the two stressors: cold water for CUS and wet bed for uCMS. The time needed to achieve the proper water volume is dependent on the water pressure, the tube diameter and technical details (valve stays open 1~2s after the plug turns off).

In the case of wet bed, 1000 mL was the necessary volume (corresponding to t=39 s) to fill in the cages containing bedding. For cold water, for the CUS protocol, 500mL of water (corresponding to t=17 s) were enough, as the cages do not contain bedding.

After the development of the water system the software was programmed and 4 different possibilities for water supply were included: 500mL=17s, 750mL=27s; 1000mL=39s and 1200mL=46s, to make the system more flexible.

3.2.2 Partial automated stressors (accessories)

Confinement to a restricted space

Two space restrictor versions were developed, one in metal and another in acrylic. Preliminary tests with animals revealed that the metal version could hurt the animals and as such we chose the acrylic version. Adding to that, the metal devices were heavier and less practical (Photo 11). Additionally, some alterations were necessary to get closer to the manual stressor. The number of the "respiratory-holes" was reduced to better mimic the original conditions and to diminish the price of the device. The size of the "manipulation-holes" was also redefined to avoid conflict with the water bottle placement (Fig 5).

The big advantage of this accessory is to reduce the time spent to perform the test and avoid having to manipulate the animals during the procedure. This new process takes 30 seconds to put one line of the rack (6 cages - 18 animals) in the confinement stressor. On the other hand, the time needed to do the same stressor in a manual way is 360 seconds.

To assess the effect of using a plastic box (manual confinement) or an acrylic restrictor (automated confinement) on the animals, acute CORT levels were measured after the procedure. CORT was collected immediately after the exposure to the acute stress, from 6 animals: 3 in the cage exposed to the manual stressor and the other 3 in another cage exposed to the automated stressor.

Results did not show statistical differences between the two procedures ($t(10)=0.1972$, $p=0.8476$), suggesting that the 2 procedures were able to induce the same kind of physiological response, as illustrated in Fig 6.

This last stressor was the only one to have an assessment of the CORT levels because it was the only stressor that had a completely different action mechanism. The other stressors were not significantly different to provoke changes in the animals' response and so, it was not necessary.

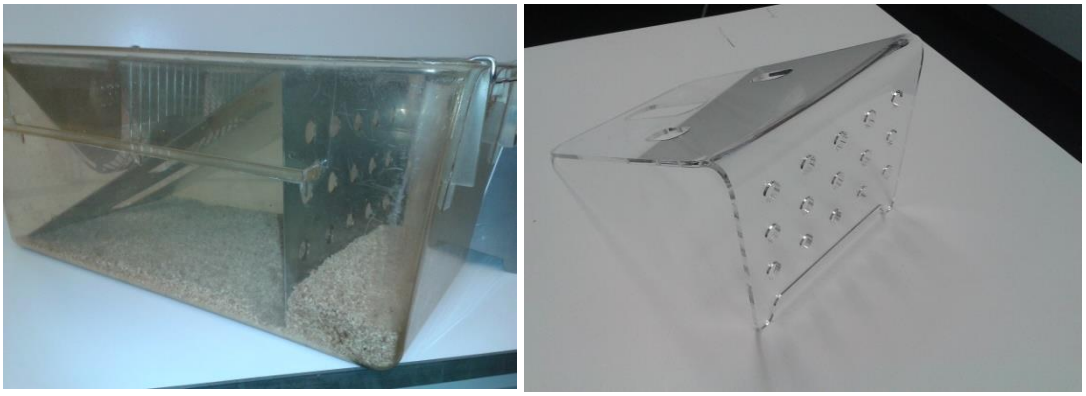

Photo 11. Accessories for partial automated stressors – space restrictor in metal (left) and in acrylic (right)

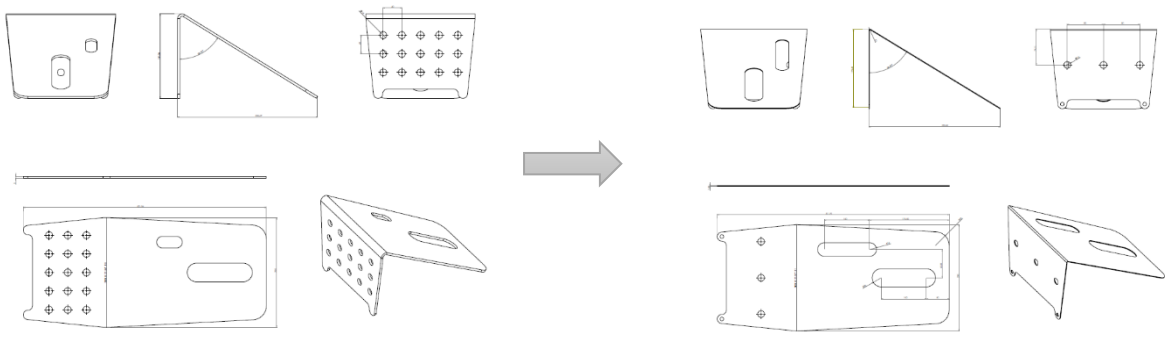

Figure 5. Scheme of two versions of partial automated stressor – space restrictor, from the oldest (left) to the new (right).

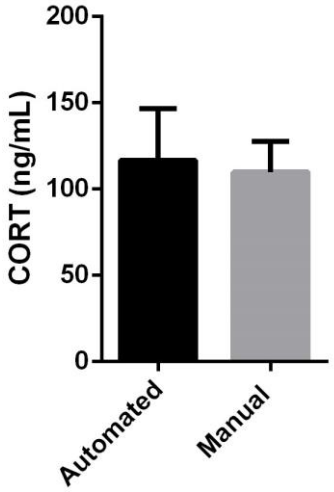

Figure 6. Corticosterone levels measured in the serum of rats exposed to a manual confinement procedure and to an automated confinement procedure. Abbreviations: CORT, corticosterone; Automated, exposure to an automated stressor; Manual, exposure to a manual stressor.

Food deprivation followed by exposure to inaccessible food (additional stressor)

In order to build the food restrictor, some prototypes of this accessory were designed. None of the versions let the animals feed properly, first because the material was thick and the distance between the grid and the restrictor was too big. Then, because the structure itself covered the access to food (**Photo 12**). A final version was developed, but unfortunately this last version was not ready at the time of the pilot study. Future work is being developed to test the final version of this stressor. Due to this, this stressor was performed just like in the manual protocol in the pilot study presented in this thesis.

Photo 12. Accessories for partial automated stressors – food restrictor from the oldest to the new version (left to right).

3.3 Pilot study: Testing an uCMS protocol with animals in the automated rack

After development and optimization of the equipment and the stressors, a comprehensive study was made. For that, we performed a complete uCMS protocol (pilot study) and analyzed several dimensions to validate the presented prototype.

3.3.1 Behaviour dimensions

The uCMS protocol typically produces deficits in 3 behavioural dimensions commonly affected in depression: anxiety, mood and cognition(5). To understand the validity of this new approach it is crucial to do an extensive analysis of the dimensions affected in order to compare with the manual protocol.

Mood

SDT and SPT were performed to measure anhedonic behaviour (**Fig 7** and **Fig 8**, respectively) in the 6th week.

In the SDT, CT groups were compared to uCMS and auCMS animals ($F(2,20)=3.930$, $p=0.0364$); uCMS presented significantly lower preference for sweet pellets comparing to control group ($p=0.0353$), the automated stress also caused a decrease in the sweet preference however not reaching statistical significance ($p=0.2162$). This decrease was tendentiously reverted by antidepressant treatment ($t(7)=1.205$, $p=0.2673$; **Fig 7**).

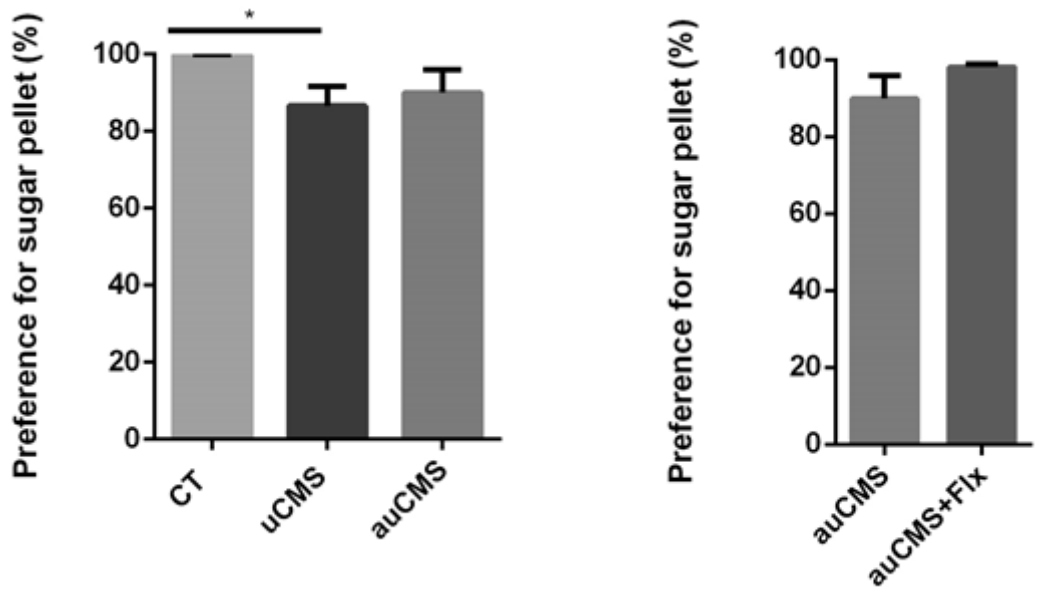

Figure 7. Assessment of anhedonic behaviour through the SDT at the 6th week of the uCMS protocol. The groups exposed to this anhedonic behavioural test were control animals, animals exposed to the uCMS and auCMS protocols as well as auCMS animals treated with fluoxetine. Abbreviations: CT, control; uCMS, manual stressed animals injected with vehicle; auCMS, automated stressed animals injected with vehicle; auCMS+FIX, automated stressed animals treated with fluoxetine. Data is presented as mean ± SEM. * p<0.05; ** p<0.01; **** p<0.0001. n= 4-10 animals per group.

In the SPT, only uCMS produced a reduction in the sucrose preference when compared to the CT group ($F(2,23)=5.858$; CT vs uCMS: $p=0.0084$ and CT vs auCMS: $p=0.8149$). Treated animals show no difference in their sucrose preference when comparing to the auCMS group ($p=0.7045$ in $t(9)=0.3915$; **Fig 8**).

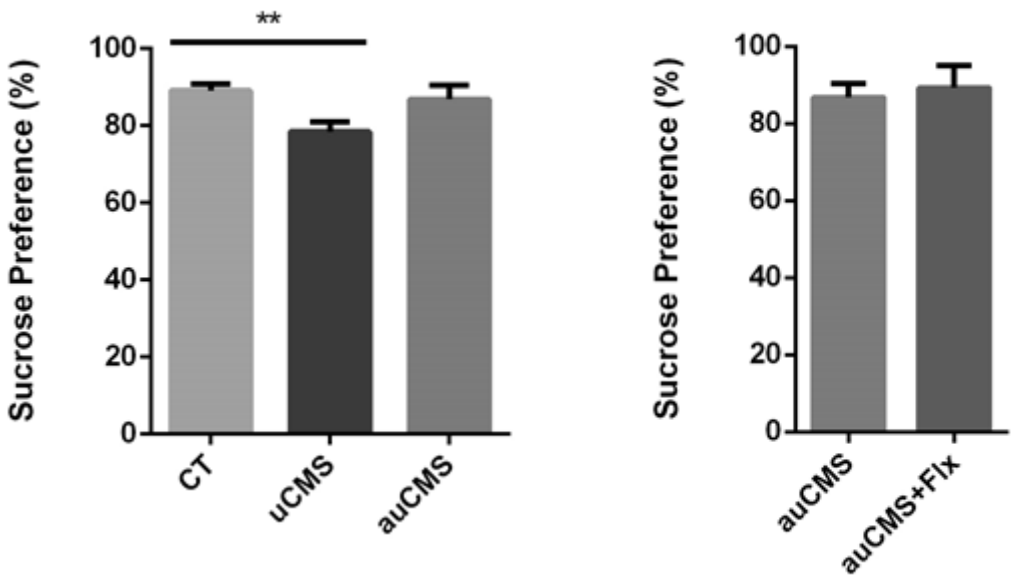

Figure 8. Assessment of anhedonic behaviour through the SPT.. The groups exposed to this anhedonic behavioural test were control animals, animals exposed to the uCMS and auCMS protocols as well as auCMS animals treated with fluoxetine. Abbreviations: CT, control; uCMS, manual stressed animals injected with vehicle; auCMS, automated stressed animals injected with vehicle; auCMS+FlX, automated stressed animals treated with fluoxetine. Data is presented as mean ± SEM. * p<0.05; ** p<0.01; **** p<0.0001. n= 5-10 animals per group.

Learned-helplessness was evaluated through the FST at the 7th week. In this test (**Fig 9**), both uCMS and auCMS-exposed animals spent more time immobile when compared to controls ($F(2,23)=3.518$, $P=0.0464$); auCMS-exposed animals show a worse performance comparing to the CT group ($p<0.0001$), however uCMS could not reach statistical significance ($p=0.1680$). Treated auCMS-exposed animals did not show differences in immobility time when compared to the non-treaded auCMS group ($t(10)=0.4087$, $p=0.6914$; **Fig 9**).

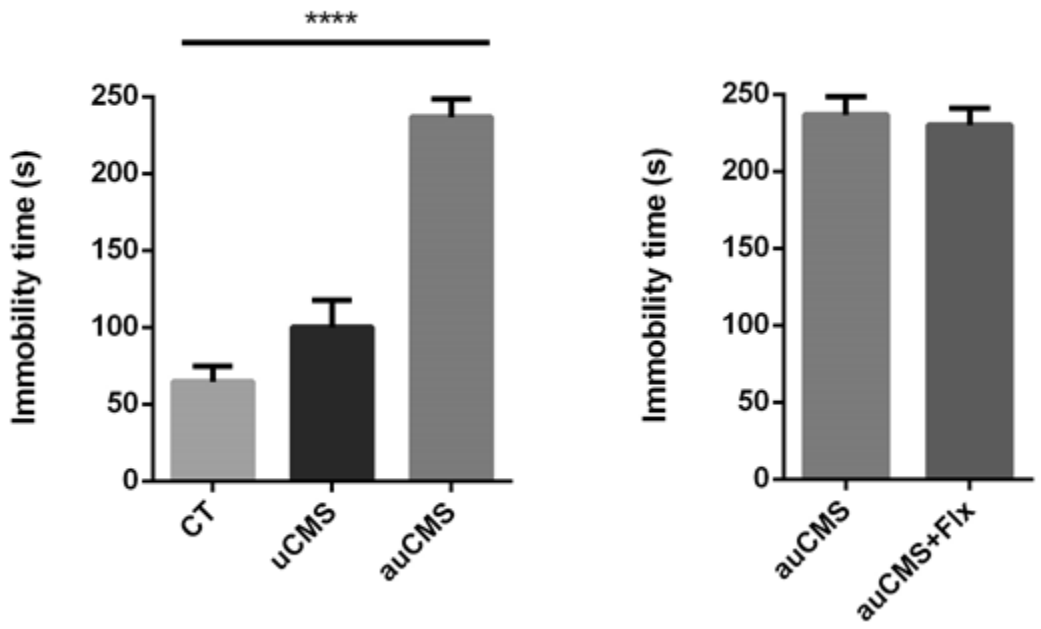

Figure 9. Assessment of mood dimension through the FST. The groups exposed to learned helplessness were control animals, animals exposed to the uCMS and auCMS protocols as well as auCMS animals treated with fluoxetine. Abbreviations: CT, control; uCMS, manual stressed animals injected with vehicle; auCMS, automated stressed animals injected with vehicle; auCMS+FlX, automated stressed animals treated with fluoxetine. Data is presented as mean ± SEM. * p<0.05; ** p<0.01; **** p<0.0001. n= 6-10 animals per group.

Cognitive function

Cognitive function was assessed through the evaluation of short-term memory (STM) and long-term memory (LTM) in the NOR test at the 7th week. When compared to CT animals, STM is impaired in both uCMS and auCMS-exposed animals (F(2,23)=0.2924, p=0.7492), but only the auCMS group reaches statistical significance (CT vs uCMS animals: p=0.0943; CT vs auCMS: p=0.0458; **Fig 10**). Animals exposed to auCMS and injected with fluoxetine (**Fig 10)** did not revealed any differences regarding cognitive impairments (t(8)=0.1029, p=0.9206) in STM.

Regarding LTM, a decrease in discrimination index was also observed in the uCMS and auCMS-exposed animals when comparing to the CT group F(2,23)=0.1530, p=0.8590; **Fig 11**). Statistical differences were found between CT and auCMS groups (CT vs uCMS: p=0.1203; CT vs auCMS: p=0.0015). The auCMS treated animals showed a tendency to have a higher discrimination index compared to non-treated animals (t(8)=0.9734, p=0.3589; in **Fig 11).**

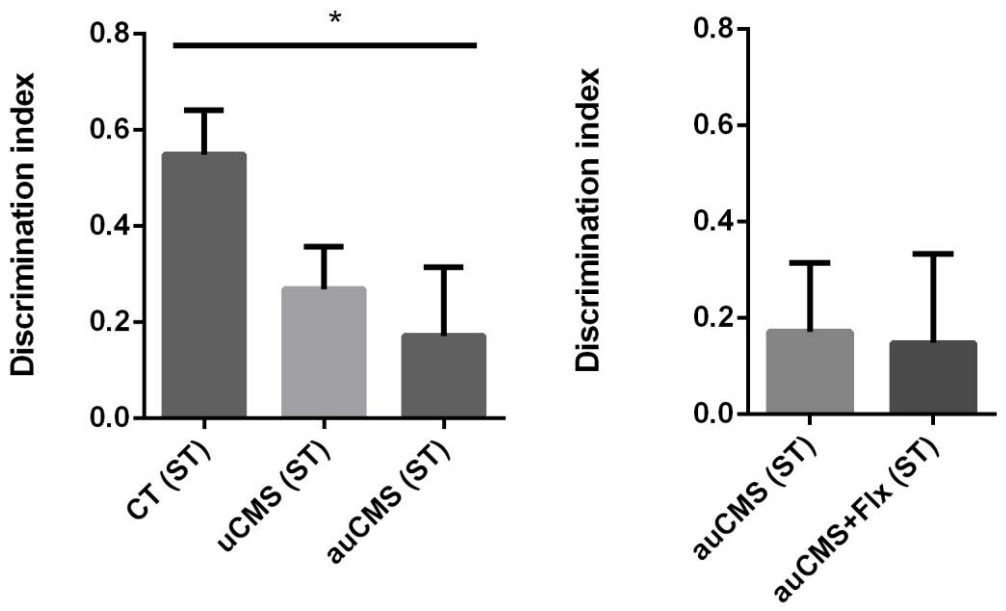

Figure 10. Assessment of short-term (ST) memory. The groups subjected to the NOR test were control animals, animals exposed to the uCMS and auCMS protocols as well as auCMS animals treated with fluoxetine. Abbreviations: CT, control; uCMS, manual stressed animals; auCMS, automated stressed animals injected with vehicle; auCMS+Flx, automated stressed animals treated with fluoxetine. Data is presented as mean ± SEM. *p<0.05; ** p<0.01. n= 4-10 animals per group.

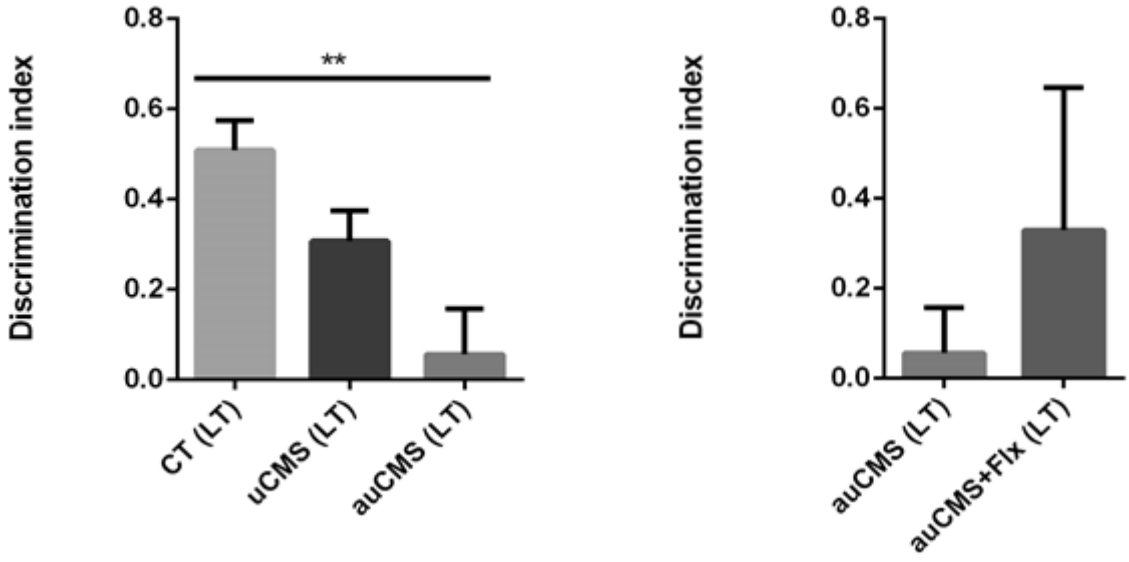

Figure 11. Assessment of long-term (LT) memory. The groups subjected to the NOR test were control animals, animals exposed to the uCMS and auCMS protocols as well as auCMS animals treated with fluoxetine. Abbreviations: CT, control; uCMS, manual stressed animals; auCMS, automated stressed animals injected with vehicle; auCMS+Flx, automated stressed animals treated with fluoxetine. Data is presented as mean ± SEM. * meaning p<0.05; ** meaning p<0.01. n= 4-10 animals per group.

Anxiety-like behaviour

To conclude, anxiety-like behaviour was assessed through the NSF test at the 7th week. Control animals displayed lower latency to feed when compared to both uCMS and auCMS-exposed animals ($F(2,23)=0.4879$, $p=0.0774$; CT vs uCMS: $p=0.0105$ and CT vs auCMS: $p=0.0005$; **Fig 12**). In line with the other tests, fluoxetine treatment could partially revert the phenotype of the auCMS-exposed animals, although not reaching statistical significance (**Fig 12**; $t(10)=1.817$, $p=0.0992$).

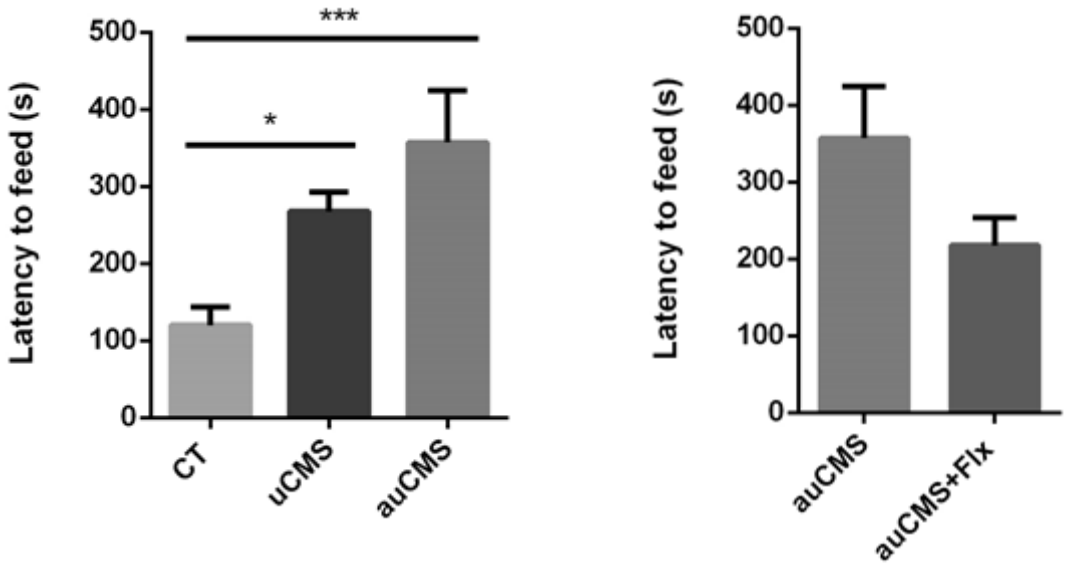

Figure 12. Assessment of anxiety-like behaviour. The groups subjected to the NSF test were control animals, animals exposed to the uCMS and auCMS protocols as well as auCMS animals treated with fluoxetine. Abbreviations: CT, control; uCMS, manual stressed animals; auCMS, automated stressed animals injected with vehicle; auCMS+FLX, automated stressed animals treated with fluoxetine. Data is presented as mean ± SEM. *P<0.05; ***P<0.001. n= 6-10 animals per group.

3.3.2 Corticosterone levels

Previous studies have shown that exposure to uCMS induces a disruption in the HPA Axis (26). Therefore, corticosterone levels in the blood serum are a valuable predictor of stress reactivity in the animals. Rats present a corticosterone peak at the beginning of their activity period that corresponds to the night period. Moreover, chronic stress exposure is known to disrupt the circadian pattern of corticosterone secretion (90,91).

At the 6th week of the uCMS protocol, corticosterone levels were measured. Differences between the hormone levels in nadir and zenith were assessed ($F(2, 46) = 9.810$, $p=0.0003$).

Control animals presented a functional circadian regulation of the corticosterone production (Nadir vs Zenith: p=0.0026). On the other hand, a disruption of the circadian rhythm of corticosterone production was observed for the two protocols of chronic stress: uCMS and auCMS (CT vs uCMS: p=0.0342 and CT vs auCMS: p=0.9765).

Animals exposed to uCMS and auCMS showed increased basal levels of corticosterone (nadir, N) when compared to CTs; although in the case of auCMS the differences did not reach statistical significance ($F(2,23)=8.126$, p=0.0021; CT vs uCMS: p=0.0218; CT vs auCMS: p=0.0802; Fig 13). Fluoxetine-treated animals could significantly restore the functional circadian regulation of the auCMS-exposed animals ($F(1,20)=5.409$, p=0.0307; auCMS vs auCMS+Flx: p= p=0.0365; Fig 13).

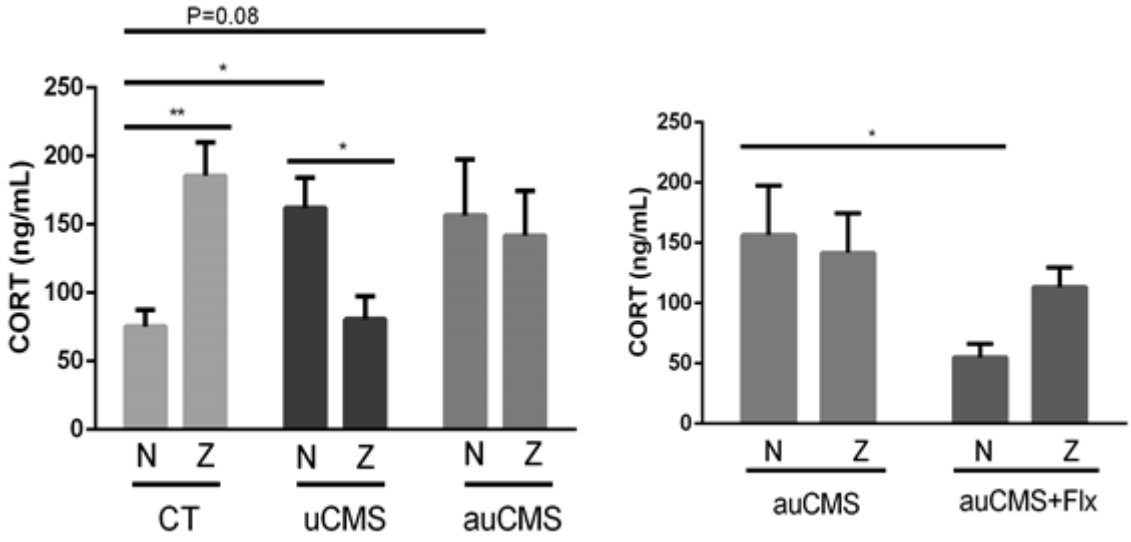

Figure 13. Corticosterone levels measured in the serum of rats between 8:00 and 9:00 am (basal levels; nadir, N) and between 8:00 pm and 9:00 pm (peak levels; zenith, Z). This figure shows corticosterone levels of manual-stressed animals, automated-stressed animals, non-stressed animals as well as fluoxetine-treated animals. Abbreviations: CT, control; uCMS, manual stressed animals; auCMS, automated stressed animals injected with vehicle; auCMS+FlX, automated stressed animals treated with fluoxetine. Data is presented as mean ± SEM. *p<0.05; **p<0.01. n= 6-10 animals per group.

3.3.3 Cellular alterations - proliferation

Additionally we assessed the proliferation levels in the hippocampal DG. It is known that animals exposed to uCMS show a decrease in cellular proliferation in the DG. This phenotype is reverted after antidepressants treatment. As such, we evaluated proliferation in auCMS treated and non-treated animals(63,64).

The number of proliferating cells assessed where dependent of the ones available on the tissue collection, since some tissue slices were damaged during the process.

Using immunofluorescence techniques with BrdU staining (one injection before the sacrifice), it was possible to see a significant increase in the number of proliferating cells ($t(32)=2.505$, $p=0.0175$) of the auCMS-treated group when compared to the auCMS non-treated group (**Fig 16**).

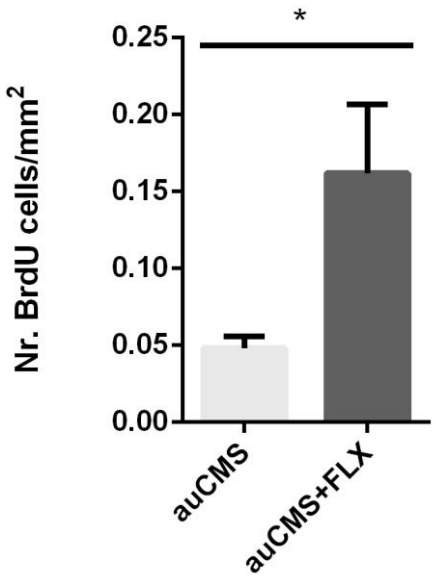

Figure 14. Number of proliferating cells in the dorsal dentate gyrus of auCMS-exposed animals untreated and treated with fluoxetine. Abbreviations: auCMS, automated stressed animals injected with vehicle; auCMS+FlX, automated stressed animals treated with fluoxetine. Data is presented as mean ± SEM. *$p<0.05$. n=3 animals per group, 3-11 neurons per animal.

3.3.4 Cellular alterations – Neuronal morphology in the hippocampal DG

Apart from behaviour analysis other approaches are also relevant to characterize the uCMS model. Therefore, brain tissue was obtained after sacrifice and it was analyzed to assess cellular alterations. Neurons morphology was assessed in the hippocampal DG, since it is a region known to be affected by chronic stress exposure; a shrinkage of the dendrites and a reduction in the dendrites complexity is seen as a consequence of uCMS exposure, with both features being reverted after ADs treatment(63).

Data obtained by 3D reconstruction of dorsal DG neurons is presented in **Fig 14**.

Surprisingly, no statistically significant differences were observed in the total dendritic length of dorsal DG neurons between CT and chronic stress-exposed animals (CT vs uCMS:

p=0.9270; (F(2,92)=3.034, p=0.0529). Regarding dorsal DG neurons of the auCMS group, no differences were also detected in the dendritic length when compared to control animals with a p=0.9794 (F(2,92)=3.034, p=0.0529; **Fig 14**).

As expected, fluoxetine-treated animals presented a higher dendritic length of dorsal DG neurons when compared to the auCMS group, although not reaching statistical significance (t(56)=1.328, p=0.1897; **Fig 14).**

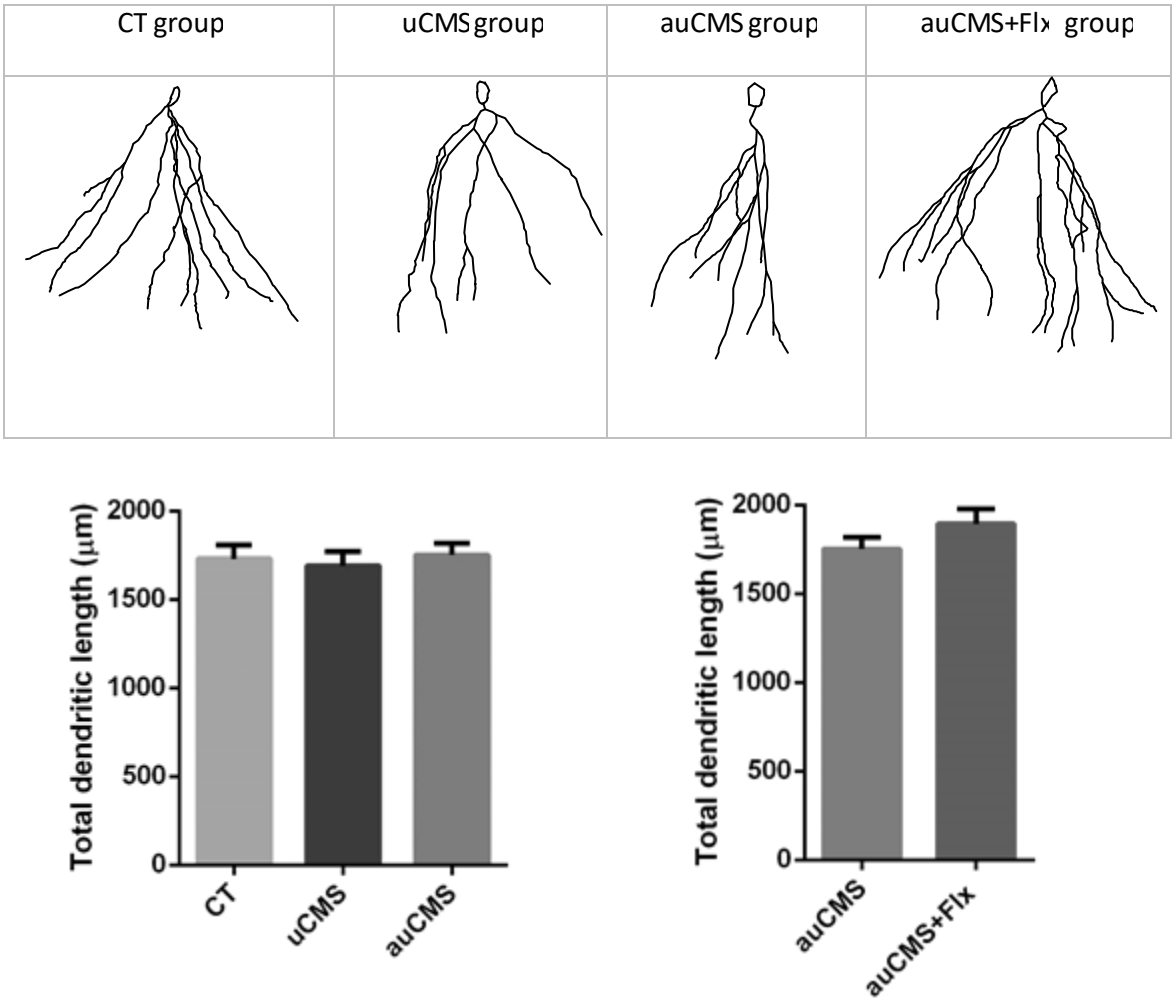

Figure 15. Morphological analysis (total dendritic length) of neurons from the dorsal hippocampal dentate gyrus of control non-stressed, manual-stressed, automated-stressed and fluoxetine-treated animals. A representative image of neurons from the different groups are shown above the graphs. Abbreviations: CT, control; uCMS, manual stressed animals; auCMS, automated stressed animals injected with vehicle; auCMS+FlX, automated stressed animals treated with fluoxetine. Data is presented as mean ± SEM. n= 3-10 animals per group.

Regarding dendritic arborization, sholl analysis was performed to assess the complexity of neurons (**Fig 15**). Again, no statistically significant differences were found between groups ($F(2,1564)=1.788$, $p=0.1676$). Additionally, a comparison between auCMS treated animals and auCMS non-treated animals showed an increase of the complexity of dendrites of neurons in the dorsal DG of auCMS treated animals (**Fig 15**). However, significant differences were not found between the two groups ($F(1,951)=2.051$, $p=0.1524$).

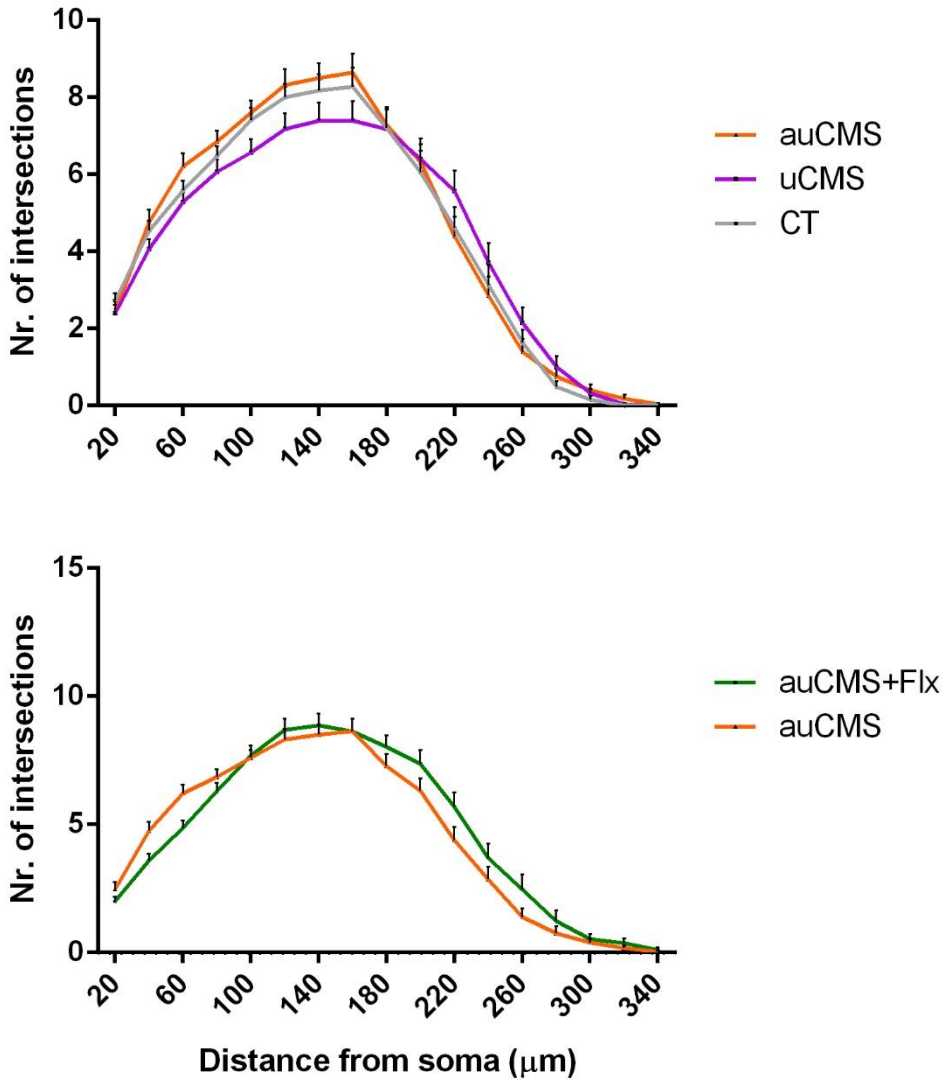

Figure 16. Morphological analysis – Sholl analysis of neurons from the dorsal dentate gyrus of control non-stressed, manual-stressed, automated-stressed and fluoxetine-treated animals. Both treated and non-treated groups were analyzed. Abbreviations: CT, control; uCMS, manual stressed animals; auCMS, automated stressed animals injected with vehicle; auCMS+FlX, automated stressed animals treated with fluoxetine. Data is presented as mean ± SEM. n=3-5 animals per group, 6-12 neurons per animal.

4. DISCUSSION

An increasing interest in **standardizing** scientific protocols and tools has been emerging among the scientific communities.

Clinical devices or equipments for human samples processing are upgraded to achieve more **accurate, rapid and reproducible** results (92,93). To a lesser extent, this has also been happening for pre-clinical research. Along this discussion I will try to cover some of the attempts made in this field to standardize preclinical animal research.

Researchers have pointed out the importance of both system and biologic validation in order to use robotized approaches in the laboratory (94). In line with that, in this validation study, we started by individually testing the stressors and then we performed a full validation of the uCMS protocol.

4.1 Equipment development and optimization

Besides the reliability of this chronic stress model seen in independent research groups, the biggest limitation of the model is still the **lack of reproducibility** among sets and laboratories (79). Literature regarding the automatization of depression protocols is practically non-existent. Two big reasons may be referred as potential causes for this lack of reproducibility - several **variability sources,** as well as difficulty of **processes' implementation**.

I will start by analysing the main contributors of procedure difficulties(79):

1) Labour-intensity work and demand of time

To overcome these, we begun by building a mechanical structure (e.g. open 3 lids at once) that reduces the labour-intensity and allows a faster manipulation of the cages for stressors implementation and for cleaning activities. This type of easy-manipulation strategies as already been described in pre-clinical protocols(95).

Looking at the protocol itself we identify particular stressors, like tilted-cage (TA) and damp bedding (TA) which are very physically demanding and time-consuming. Through our modifications, it is possible to remotely control the system and implement the stressors without the presence of the experimenter in the animal facility. Confinement (PA) is another stressor that implies much work and time. This stressor can be implemented through different ways, like restrain one animal in a cylinder(96,97) or place three animals inside a plastic box with restrained space and air(5,59) – the one we used to perform uCMS. As such, we decided to develop a more practical alternative that wouldn't involve the manipulation of the animals, another important

point to be discussed ahead. Our proposed method it is not a totally automated stress, although it decreases the time per cage from 60s to 5s as well as the work involved.

2) Space constraint

Since most of the protocols are performed with a big number of animals, the space required to perform tilt-cage or confinement may be a constraint for several laboratories. The prototype presented here can reduce it to the space of a regular rack since all the cages are incorporated in the equipment.

Regarding the variability between laboratories no clear factors have been previously and systematically identified. However, some speculations have been made regarding the causative factors (77,79,98). Next, I discuss these possible factors more in depth:

1) Divergences between protocols

Design variations such as permutations between stressors, time of testing or logistics seem to play an important role in the uCMS outcomes. To overcome these issues some protocol alterations were implemented, the automation of the tilted-cage stressor (TA) allow us to place the animals with the same inclination (previously programmed), at the same time. Also, the original damp bedding stress (TA) would often leads to a supply of uneven quantities of water, something that does not occur with our auCMS protocol.

Apart from that, it is still important to ameliorate some issues that could have contributed to the divergences. For instance, the control computer had the lights of the screen on because it was placed in the same room as the equipment, this illumination could have interfered with the protocol and could have influenced the light-cycle of the animals (it must be corrected in further protocols).

Some opportunities may appear by taking advantage of the equipment features because it allows to perform the protocol in an automated equipment it is possible to programme the system to introduce stressors in the active period of the animals (at night). This approach could be explored in order to better understand the influence of performing stressors in diurnal/nocturnal period for the effectiveness of depression-induction protocol, in fact it was observed that rats who were stressed during their active period (night) did not display signs of depressive and anxiety-related behaviours(98). Another interesting topic that could be assessed is the impact of different stressor-permutation levels along active or non-active period may have on the effectiveness of depression-induction protocol.

2) Differences in housing conditions

Stressed and control animals are housed in different rooms. An important parameter to be monitored in the housing conditions is the ventilation since the circulation of pheromones as well as other external odour stimuli may interfere with the animal model induction and with the behaviour of control animals(99). These parameters did not suffer any alteration since this already happened in our animal facilities.

It is relevant to say that the automated equipment is not silent (e.g. engine activity for tilted cage) and it is not advisable to keep it in a room near the control animals. To solve this question a noise isolation box for the engine may be considered in the future.

Other research groups have been exploring the relevance of the housing conditions for the development of depressive-like behaviour, by researching alternative solutions to house the animals in a more naturalistic way. Standard housing conditions that increase sensory, cognitive, motor and social stimulation are being proposed for rats and mice(95). A particular example is PhW - PhenoWorld, a different paradigm that was created to better mimic normal environmental conditions(100).

3) Influence from the operator

Alternative methods to perform behavioural tests in a more fluent and integrated manner are being studied. An example of that occurs in PhW paradigm where animals are video-taped in their home cages in order to measure sleep duration and social interaction without disturbing them(100). Another example is the growing use of ultrasonic vocalizations(85,101), a methodology where animals are also assessed in their housing environment without the need of experimenter manipulation(101), these new methodologies will allow the achievement of more realistic conditions.

Similarly, stress induction protocols may also be influenced by the operator, namely regarding different handling experimenters or intensities. Indeed, stressed animals are more handled than control ones which may raise behavioural alterations that are impossible to control. When compared to the original, our protocol offers a solution to reduce this problem. For example, in our new proposed confinement (PA) we eliminate the need to manipulate the animals.

Another key issue of behavioural tests is the experimenter bias, which is influenced by subjective ratings. Indeed, scoring criteria vary within observers despite similar instructions(102). In that concern, an increasing number of equipments for rodents' activity measurement is being developed. These devices enable to analyze FST, NOR or social defeat with a faster data

acquisition as well as the production of more accurate results as the subjective operator influence is not present (102,103). It is relevant to say that our behavioural tests, particularly FST and NOR, where measured using these type of equipments.

These softwares and devices for the automatic measurement of behaviour phenotyping have been patented, highlighting the growing importance and interest on this area(104,105).

4) Animal variances

Intrinsic inter-animal variances are much difficult to control. Even among the same strain it is possible to see differences between suppliers, due to the genetic background or rearing procedures. Additionally, the composition of the gut microbiota, which may vary according to the housing conditions, has been increasingly seen as an influencing factor for the development of depressive and anxiety-like behaviour. Supporting this idea is the common observation of co-morbidities between GI disorders and MD(106) as well as associations between severity of gastrointestinal symptoms and abuse experiences (related to mood disorders)(106,107).

Overall, our prototype is capable of improving the main operating difficulties found in the original uCMS protocol as well as several variability sources. These improvements are of particular usefulness because uCMS protocol is very long and demanding.

In sum, with the construction of this equipment we intend to improve uCMS protocol effectiveness through a more rigorous and discerning approach, in line to what has been published in the literature. In fact, the creation of standardized methods is crucial to clearly identify putative sources of variability and achieve reproducibility. Soon, a patent application will be filled as a result of the development of this equipment.

4.2 Pilot study phase

After accomplishing the aim of construction and optimization of the automated equipment, we proceeded to a pilot study in order to validate it. In this pilot study (1st protocol developed with the equipment) a small cohort of animals was exposed to a six-week uCMS protocol.

In this study we wanted to compare the manual uCMS protocol results with the outcomes obtained for the automated uCMS protocol, here described. For that, we showed some preliminary results regarding the comparison of both protocols, including behavioural outcomes (face validity), treatment efficiency (predictive validity) as well as molecular and cellular findings (construct validity).

Indeed, uCMS protocol has been previously validated as one of the most reliable models for depressive-like behaviour induction due to its aetiology validity as well as its completely fulfilment of the validation criteria for animal models.(5,70,77)

Depression is a multi-dimensional disorder that presents high comorbidity with anxiety(6) as well as cognitive impairments(7). In fact, several behavioural dimensions (mood, anxiety and cognition) are often impaired in depressed patients and animal models of this disease. Consequently an extensive behavioural evaluation with a broader perspective must be applied in order to get closer to what is seen in clinics(5).

In this context, several behavioural tests were performed to evaluate the impact of chronic stress exposure in the 3 dimensions affected by depression, through the following sequence: SPT > SDT > NOR in the 6th week and NSF > FST in the 7th week of the protocol. In the 6th week of the uCMS protocol the most sensitive tests were performed and in the 7th week the most aggressive ones were performed, to not cause any interference between them.

The SPT is known as a gold standard-test to measure anhedonic behaviour(79). This test is not extremely sensitive and requires relatively big sets of animals to obtain consistent results, as some animals do not perform the test. Due to that, we decided to use the SDT as a complementary test, since this new approach was developed to assess the same behavioural domain as the SPT - anhedonia. Both manual and automated uCMS protocols induced slightly lower preference levels for sucrose solution and sweet pellets; however, statistical differences were only reached with uCMS manual protocol. According to the literature (77,108) and to previous experiments of our team, tests to evaluate anhedonia are challenging and the intra-group variability may be a concern. As previously referred, in this case, the small number of

animals may be an explanation for the lack of statistical differences between controls and auCMS-exposed animals.

The FST is a learned helplessness test developed by Porsolt(109), where a passive immobility behaviour is measured. This test has a more extreme/aggressive nature, in fact some authors use it as a depression model(110). This is also a gold standard test to assess depressive-like behaviour in rodents but still controversial as some authors argue that immobility is largely dependent on learning/memory and may be a result of habituation; also the acute efficacy of antidepressant treatment in this test does not mimic clinics (111,112). Here, the FST results showed that chronically stressed animals presented increased levels of immobility when compared to control ones. Interestingly, the auCMS-exposed animals, in particular, showed a worse performance, statistically significant from the non-stressed animals which was already reported in PhW (a new ethological enriched paradigm that tries to reduce manipulation influence) where uCMS rats from the PhW demonstrated increased levels of behaviour despair compared to the ones in standard conditions(113).

Cognitive function, specifically long term and short term memory, were assessed through NOR - a behavioural test based on object-recognition task, commonly used to study memory(86,87). As expected, our results show a reduced discrimination index of LTM and STM for both stress protocols when compared to controls. Interestingly, statistical significance was only achieved between control and auCMS group.

Anxiety levels were measured by NSF, where higher latency to feeding in the open field was used as an index of an increased anxiety-like behaviour. Previous studies report the increased levels of anxiety in uCMS animals, which was statistically confirmed in our results for both protocols (manual and automated)(5,59). Interestingly, auCMS showed a “stronger” phenotype since differences between CT vs auCMS were more evident than CT vs uCMS.

Despite the lack of statistical differences between auCMS vs CT for SDT and SPT, all the other tests show severe results between auCMS vs CT than uCMS vs CT. As stated before difficulties in performing these two behavioural tests may be an explanation, however it would be interesting to complete this analysis with USVs, known to be related with anhedonic behaviour.

Altogether our results suggest that all the 3 behavioural dimensions are impaired in animals subjected to the automated exposure to chronic stress, similarly to what is observed with the manual chronic stress-exposure protocol(5). Although these results must be confirmed in another set of animals, face validity of the auCMS protocol can be considered fulfilled.

Fluoxetine administration was used to assess predictive validity. This antidepressant is widely used in the clinics and belongs to the Selective Serotonin Re-uptake Inhibitors (SSRIs).

Fluoxetine was chosen because it is known to improve depressive symptoms in humans and also reverses depressive-like behaviour signs in animal models of depression. Importantly, previous studies have shown the ability of fluoxetine to revert depressive-like behaviour in animals exposed to uCMS(5,85). Similarly, our results show a slightly better performance in the behavioural tests of treated animals when compared to non-treated, suggesting a predictive validity of the automated protocol. Again, the lack of statistical significant results may be due to the low number of animals used in this pilot study. Thus, these results should be confirmed in the future with a larger set of animals.

Corticosterone measurement are usually a molecular assay used to verify the efficacy of stress exposure protocols(85,114) since chronic stress leads to a disruption of the normal circadian corticosterone production(90). The circadian rhythm of corticosterone in control animals is usually characterized by low levels in the morning and a peak at the beginning of the night phase (animals' active phase)(91). The HPA-axis is one of the systems responsible for controlling GRs release; after chronic stress exposure animals display an hyperactivity of this system which leads to a deregulation of the GC secretion-pattern into the blood (90,115). Here, we observed an alteration of the circadian regulation of corticosterone secretion in chronic stressed animals and a reversion of the corticosterone levels to normal patterns after treatment with fluoxetine. These results confirm the successful stress induction of the animals using the automated protocol (as it is observed in the manual protocol) and a reestablishment of the normal circadian rhythm after antidepressant treatment of auCMS group.

Concerning cellular alterations, neurons morphology in the dorsal hippocampal DG was evaluated using Golgi-staining.

Previous studies of uCMS-triggered depressive-like behaviour showed alterations in the neuronal morphology in the hippocampal DG. Dendritic atrophy of dorsal neurons was seen through the reduction of dendritic length; also a decrease of ramifications number and alterations in dendritic distribution was reported through 3D sholl analysis (morphological characterization of neurons based on concentric circles positioned at radial intervals of 20 µm)(47,48,66). Our results failed to show changes in the dendritic length and in the complexity of dorsal DG neurons of animals exposed to both protocols of chronic stress. As the number of animals used for these analyses was very small, at the moment the results are inconclusive for both stressed groups and so, it is not possible to take further conclusions. Fluoxetine treatment increased the length and complexity of neurons from animals of auCMS protocol, although not reaching statistical significance. Our results are thus in line with the literature since a reversion of the induced morphological changes was observed in neuromorphological studies in the hippocampus and PFC(63,82). Similarly, other ADs, like TCA and MAOIs were shown to restore dendritic plasticity

and thus it is expected that they would also be able to reverse the effects of auCMS(63,64) – an interesting experiment that could be included to enrich this work.

In the future, it will be important to analyze other regions known to be affected in depression, like the prefrontal cortex. Deficits in executive function characterize stress-related disorders(5) and post mortem analysis of depressed patients show morphological changes in neurons and glial cells of PFC. Also, preclinical studies show that chronic stress impair functional synaptic plasticity (LTP reduction) in the hippocampal-PFC connections(33,97). Taking that in mind, PFC is a frequently explored region in depression models and it will be relevant to address in this context in the future. Additionally, we expect also to analyze other parameters related to dendritic and synaptic plasticity such as spine numbers and spine morphology, since it was reported that chronic stress reduce spine density in hippocampus and PFC. (22,59,63). These approaches will help us to better characterize and validate the cellular/plastic alterations induced by chronic stress exposure in our proposed method.

Previous work had shown that uCMS protocol have an impact on cellular proliferation; stressed animals are known to present reduced levels of proliferation, neurogenesis and gliogenesis in the dorsal DG of the hippocampus (45,52). In fact, long-lasting, sustained remission of the impaired dimensions of depression is dependent of neurogenesis (59,66). Due to time constraints, our results only assessed whether there was a difference between fluoxetine-treated animals exposed to auCMS and the ones exposed to stress that were not treated. As expected, we observed an increase in the number of cells being generated in the dorsal hippocampal DG after fluoxetine treatment, which is consistent with the literature(59,64). Indeed, animals exposed to uCMS protocol display impaired neurogenesis with reversion after AD treatment(59,64). Although the analysis of the neuronal morphology in the DG was not conclusive, molecular results suggest a validation of the construct criteria. As such, construct validity was only partially fulfilled in this pilot study of the auCMS protocol.

In this study, we did not analyze cell fate, in further experiments it will be useful to add another marker to identify cells fate (e.g. doublecortin (DCX) for immature neurons), this suggestion could help to specify and detail our results. Another suggestion is to analyze gliogenesis which has been more recently correlated with the pathophysiology of depression (63,117).

Together, our results suggest that the auCMS protocol implemented has partially fulfilled construct validity, face and predicted validity.

According to Belzung, another parameter which is important to discuss is the right choice of the animal for the model, according to biological features(68). In fact, the strain of rats used in this work (Wistar Han rats) has been consistently described as an appropriate strain to study

depression. Additionally, responses to behavioural tests using this strain have been validated for all the dimensions affected by depression(5,77,82). Also, our laboratory has been using and validating this strain for several years and so, an internal validity is established (with reproducibility and inter-observer reliability). These findings, lead us to consider that the homological criteria was fulfilled.

In summary, our results suggest that, the automated protocol of chronic stress exposure can mimic to a large extent the manual protocol. Moreover, the automated protocol lead to more evident deficits in anxiety and depressive-like behaviour, as well as to memory impairments. The reduced influence of the operator manipulation as well as the application of slightly more intense stressors when using the automated rack are most likely playing an important role.

4.3 Main limitations

To conclude some limitations were identified. During the 6th week protocol some electrotechnical failures were detected and corrected. In the future it would be important to have a video-camera in order to monitor the protocol during 24h to assess the problems, as soon as possible. Another limitation of this work is the reduced number of animals used, in fact this small number was chosen due to the preliminary nature of the work.

The equipment being tested has other features beyond the ones that were tested. This work was mainly restricted to the uCMS protocol however, as it was explained before, it is also possible to perform a CUS protocol since it is capable of incorporating stressors from this protocol.

The development of this equipment is a crucial innovation for Bn'ML, since it will allow them to be positioned as the first company to try to standardize the depression induction protocol through the development of an automated equipment for rats. Due to that, services for pharmaceutical companies may be provided with more accuracy.

Beyond that, this innovation is fundamental for the improvement of the actual pre-clinical research approaches. Pharmaceutical companies will be able to test their drugs in a more standardized way, which is a step forward to the global implementation of this model and a powerful contributor to a better screen of novel drugs.

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