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How can cognitive enrichment revert the effects of stress?

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Mestrado em Biologia Humana e Ambiente

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Resumo

O sistema nervoso é responsável pelos processos que tornam a vida humana possível. Permite-nos pensar, sonhar e criar memórias. Controla as nossas acções mais básicas e involuntárias como piscar os olhos, respirar e manter os nossos corações a bater. Os neurónios são as unidades básicas do sistema nervoso, são células notáveis pela especialização para comunicação intercelular que apresentam. Normalmente os neurónios apresentam quatro regiões distintas: o corpo celular, dendrites, axónio e terminais pré-sinápticos. Entre a ponta de cada terminal sináptico e o ponto de contacto no neurónio seguinte há um pequeno espaço chamado sinapse. Cada neurónio pode ligar-se a cerca de 1000 a 10000 outros neurónios. Apesar de muitas destas conexões serem especializadas, todos os neurónios utilizam uma de duas possíveis formas de transmissão sináptica: eléctrica ou química. A força de ambas as formas de transmissão sináptica pode ser aumentada ou diminuída através da actividade celular. Esta plasticidade sináptica é vital para a formação de memórias e para os processos de aprendizagem. *Long term potentiation* de sinapses químicas é um dos modelos mais estudados para a formação de memórias no cérebro de mamíferos.

O sistema nervoso pode ser dividido em duas partes principais: o sistema nervoso central (SNC), que é constituído pelo cérebro e espinal medula, e o sistema nervoso periférico (SNP), que consiste nos nervos craniais e espinais e gânglios associados. O sistema nervoso central pode ser dividido em sete partes: espinal medula, medula, ponte, cerebelo, mesencéfalo, diencéfalo e os hemisférios cerebrais.

O córtex prefrontal poderá estar relacionado com a organização da informação interna e externa que é necessária para produzir comportamentos complexos. É a área cerebral que mais espaço ocupa no cérebro humano, e é maior na nossa espécie do que em outros primatas. Este facto poderá ser mais um indício de que o córtex prefrontal está envolvido em algumas capacidades consideradas inteligentes. Está também envolvido no processamento de informação cognitiva e emocional e nos processos de memória de trabalho ou

working memory (WM). A *working memory* é uma memória temporária que é bastante útil no cumprimento de tarefas.

O hipocampo é outra zona do cérebro, também muito importante na formação de memória (em especial, memória espacial) e na aprendizagem. As funções do hipocampo podem varar entre espécies, mas a maior parte dos estudos são convergentes, no que respeita a caracterização da anatomia e fisiologia desta área.

O hipocampo está ligado ao córtex prefrontal, sendo que esta conexão é feia por axónios que têm origem no subículo e terminam nos neurónios piramidais do PFC.

O stress é um aspecto comum nas nossas vidas diárias, mas ainda assim há alguma ambiguidade no que toca à sua definição. É um desafio real ou percepcionado, quer endógeno quer exógeno, que perturba o equilíbrio natural do organismo – homeostase. Os humanos e outros animais respondem a estes desafios desencadeando uma série de mecanismos neuronais, endócrinos, neuro-endócrinos e metabólicos.

O impacto do stress no cérebro tem recebida imensa atenção nos últimos anos, tanto da parte de neurologistas como de leigos. De facto, o cérebro é o principal órgão por detrás da resposta ao stress: determina o que é ou não uma ameaça, coordena as reacções que cada um tem, e altera-se, estrutural e funcionalmente como resultado de experiências *stressantes*. Uma das principais respostas ao stress é a activação do eixo HPA (hipotálamo-pituitária-adrenal).

Os danos no córtex prefrontal e no hipocampo em consequência de episódios de stress ou de stress crónico estão bem descritos.

Em 2007, Cerqueira demonstrou que a plasticidade sináptica entre o hipocampo e o córtex prefrontal diminui depois de um período de stress crónico e que os efeitos do stress neste circuito são devidos a atrofia neuronal, e não a perda de neurónios. Assim, este trabalho propôs-se a tentar compreender como é que estes efeitos do stress poderão ser revertidos. Para isso, utilizámos ratos Wistar Han como modelo animal, e criámos quatro grupos distintos: animais stressados, animais stressados que foram sujeitos a uma tarefa cognitiva, animais controlo e

animais controlo que foram submetidos à mesma tarefa. Posteriormente, avaliámos dois parâmetros em cada grupo: plasticidade sináptica e análise morfológica. Para esse efeito utilizámos um protocolo de electrofisiologia. Um estímulo eléctrico no hipocampo gera uma resposta no córtex prefrontal, e essa resposta pode ser aumentada se for induzido LTP. No entanto esse aumento (ou potenciação do sinal) não é tão elevado em animais stressados, sendo por isso uma boa medida para avaliar se o *hole board* consegue ou não reverter a perda de plasticidade sináptica neste circuito. A análise morfológica consistiu em reconstruir neurónios em três dimensões, utilizando o software Neurolucida e assim conseguir avaliar a quantidade de dendrites, o seu comprimento, e a densidade de espinhas de cada neurónio estudado.

Os nossos resultados demonstram que o treino no *hole board* foi bem sucedido, já que todos os animais aprenderam a tarefa. No entanto, os animais stressados têm mais dificuldade em aprender a tarefa. No protocolo de electrofisiologia, os resultados foram os esperados, os animais stressados demonstraram menor plasticidade sináptica, enquanto que os animais que fizeram a tarefa cognitiva demonstraram maior plasticidade sináptica, embora o aumento da resposta do prefrontal córtex não fosse tão elevado como nos controlos. Em relação à análise morfológica, os resultados também corresponderam ao esperado. Em praticamente todos os parâmetros foi visível a recuperação dos animais que treinaram no hole board, como por exemplo no número de ramos dendríticos, no seu comprimento ou ate na densidade dendrítica de espinhas.

Em conclusão, este estudo demonstrou que uma tarefa cognitiva, neste caso o hole board, pode reverter alguns efeitos do stress, incluindo a perda de plasticidade sináptica e a atrofia neuronal na conexão entre i hipocampo e o córtex prefrontal.

Palavras-Chave: Stress, enriquecimento cognitivo, hole board, electrofisiologia, correlatos morfológicos.

Abstract

It is known that stress has negative effects on our health. It is important to understand how stress affects us and how we can revert those effects. We proposed the hypothesis that some consequences of stress might be reverted through cognitive enrichment. So, using Wistar Han rats as an animal model, we formed four groups of animals: control animals, control animals that performed a cognitive enrichment task (hole-board), stressed animals, and stressed animals that performed the same task. We then evaluated two parameters on every animal: synaptic plasticity between the prefrontal cortex (PFC) and the hippocampus and morphological correlate. After analyzing the results, we could conclude that the cognitive enrichment task can, indeed, revert some effects of stress, including the loss of synaptic plasticity and neuronal atrophy.

Keywords: Stress, cognitive enrichment, hole board, electrophysiology, morphological correlates.

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1 | Introduction

1.1. Neurosciences

"Neuroscience is the multidisciplinary science that analyzes the nervous system to understand the biological basis of behavior" [1].

According to Kandel, neuroscience is the understanding of the biological basis of consciousness and the mental processes that allows us to perceive, act, learn and remember, and it is the ultimate challenge of the biological sciences [2]. The neuroscientists' intent is to explain behavior in terms of the activities of the brain and to understand how the brain can organize its millions of nerve cells to produce behavior and how are these cells influenced by the environment [2].

The nervous system is responsible for all the processes that make human life possible. It allows us to think, dream and have memories. Furthermore, it handles our most basic, involuntary actions and reactions like blinking our eyes, keeping our body at the right temperature, breathing, and making our heart beat. It is the body's manner of communicate with itself and the outside world [3].

1.2. The nerve cell

From a historical point of view, no other cell type has attracted as much attention or caused as much controversy as the nerve cell, or neuron [4]. It was not until the twentieth century that neuroscientists agreed that, like other organs and tissues, the nervous tissue is also made of these units. This finding was

delayed especially due to the complexity of the nerve cells and their extensive branching [5]. In the end of the nineteenth century, an Italian histologist, Camillo Golgi, established a staining technique based on silver nitrate that allowed qualitative and quantitative characterization of neuronal morphology – the Golgi staining, as mentioned on a Milatovic's study [6].

Although some authors defended the theory that the nervous system was a continuous nerve cell network (known as the reticular theory), it was only in the 1950s, with the advent of the electron microscopy, that the existence of neurons as discrete entities became well established (neuron theory). Ramón y Cajal used the method established by Golgi to examine nervous tissue on light microscope to argue that nerve cells are discrete entities, and that they communicate with each other via specialized contacts called synapses. These histological studies, from Cajal, Golgi and other authors, led to the consensus that the nervous system cells can be categorized in two groups: nerve cells (neurons) and supporting cells called neuroglia (or simply glia) [5].

Neurons have the same intracellular components as the other cells, in fact, no unique cytoplasmic component of the neuron discriminates it from any other cell. Neurons comprise all the morphological machinery of other cell types, the structures are similarly located and the Golgi apparatus and mitochondria, for example, were described first in neurons [4]. However, neurons are visibly notable by their specialization for intercellular communication. This quality is apparent in their general morphology, in the specific organization of their

membrane components for electrical signaling, and in the structural and functional details of the synaptic contacts between neurons [5].

Typically, the neuron has four morphologically defined regions: the cell body, dendrites, the axon and presynaptic terminals; all of these regions have a distinct role in the making of signals and the communication of signal between nerve cells [2] (Figure 1).

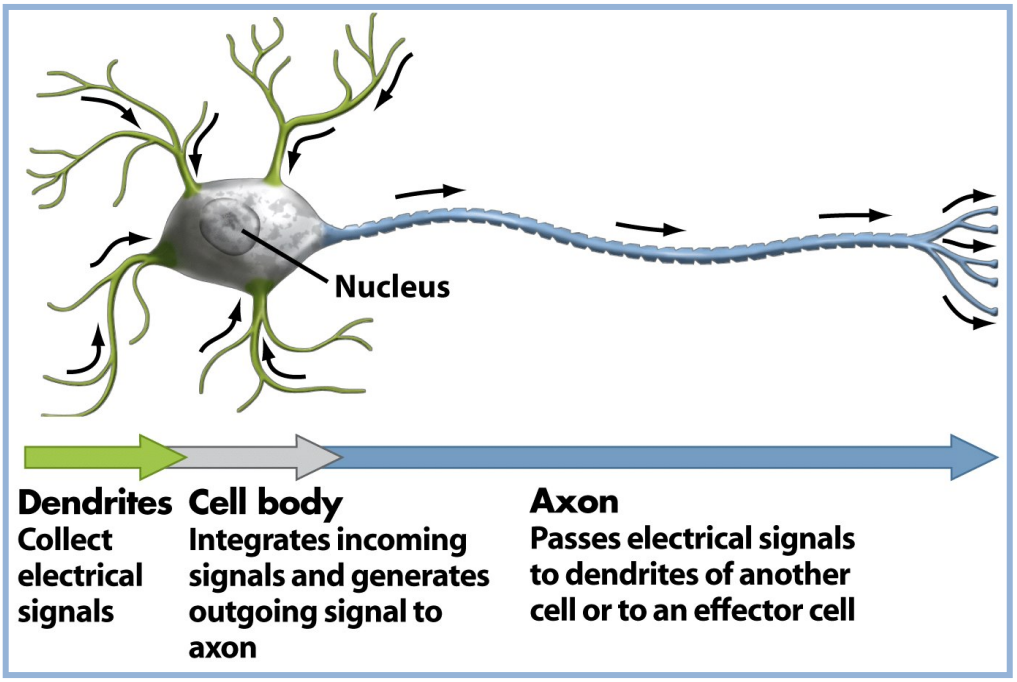


Figure 1 – The nerve cell and electrical signaling [A].

The cell body – soma - is the metabolic center of the cell. It contains the nucleus, responsible for storing the genes of the cell, along with the endoplasmic reticulum. The cell body usually originates to two kinds of processes: some short dendrites and one long, tubular axon. Dendrites are divided in a tree-like fashion and are the main machinery for the reception of

incoming signals from other nerve cells. On the other hand, the axon lengthens away from the cell body and is the main conducting element for carrying signals to other neurons. An axon can pass on electrical signals along distances ranging from 0.1mm to 3m [2] (Figure 1).

Nevertheless, neurons are not all the same; they can be excitatory, inhibitory or modulatory in their effect, and motor, sensory or secretory in their function [4]. They can also vary in shape and size (Figure 2).

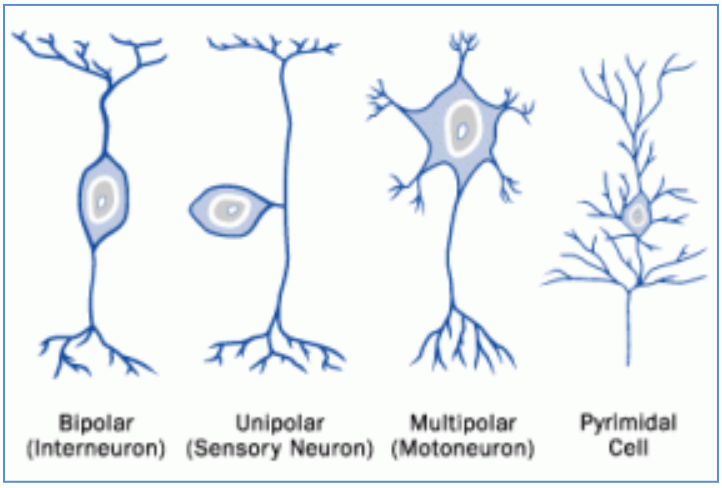


Figure 2 – Basic neuron types [B].

Connectivity between neurons

When the nervous system receives an impulse, it may respond by sending another impulse to the appropriate effectors or to another neuron, which triggers it to the next neurons, and so on. A neural impulse is triggered by a change in the neuron's electrical charge, which is the result of rapid ionic changes [7].

Even though neurons are not inherently good conductors of electricity, they have developed elaborate mechanisms to produce signals, based on the flow of ions across their plasma membranes. Ordinarily, neurons generate a negative potential, called the resting membrane potential, which can be measured by recording the voltage between the inside and outside of nerve cells [5]. The interior of a resting neuron contains more negatively charged ions than the outside of the cell, where there are more positive ions. This biased distribution of electrical energy is referred to as membrane potential and is critical to the neuron's ability to transmit an impulse along its entire length [7].

At the spot where the neuron is stimulated, the membrane becomes more permeable to sodium ions, which then rush across the membrane to the inside of the cell, momentarily producing a slightly more positive charge inside the cell relative to the outside. This change in membrane potential is called depolarization. After the neuron becomes more permeable to sodium ions, it also becomes more permeable to potassium ions. So, in the fraction of a second following the influx of sodium ions, potassium ions rush out of the cell. This exit of positively charged ions restores the negative charge inside the cell as well as on the membrane. Once depolarization has been initiated at one end of the neuron, it passes down the entire length of the neuron. For this reason, this wave of depolarization, also known as action potential, is termed "all or nothing". This complete sequence of events is known as a nervous impulse [7].

Between the tip of each axon terminal and the point on the next neuron, usually a dendritic spine or the cell body, to which the axon sends a nerve signal, there is a minute gap. It is called the synaptic cleft. The synapse (Figure 3) refers to the synaptic cleft and the areas on the two neurons that are involved in the transmission and reception of a signal [8]. Each neuron is connected via synapses to about 1000-10000 other neurons [9].

Even though many of these connections are specialized, all neurons use one of two basic forms of synaptic transmission: electrical or chemical [2]. Although most synapses use a chemical transmitter, several operate only by electrical means. Once the structure of synapses became evident with the electron microscope, differences in the morphology of chemical and electrical synapses were found. At chemical synapses, neurons are separated completely by a small gap, the synaptic cleft; there is no continuity between the cytoplasm of one cell and the next. In contrast, at electrical synapses the pre- and postsynaptic cells communicate through special channels, the gap junction channels, which serve as a connection between the cytoplasm of the two cells [2].

The most frequent mechanism for signaling between neurons is the neurotransmitter-releasing chemical synapse. Nevertheless, faster and simpler signaling can be performed with electrical synapses, specialized connections that allow ionic current to flow directly between neurons [10].

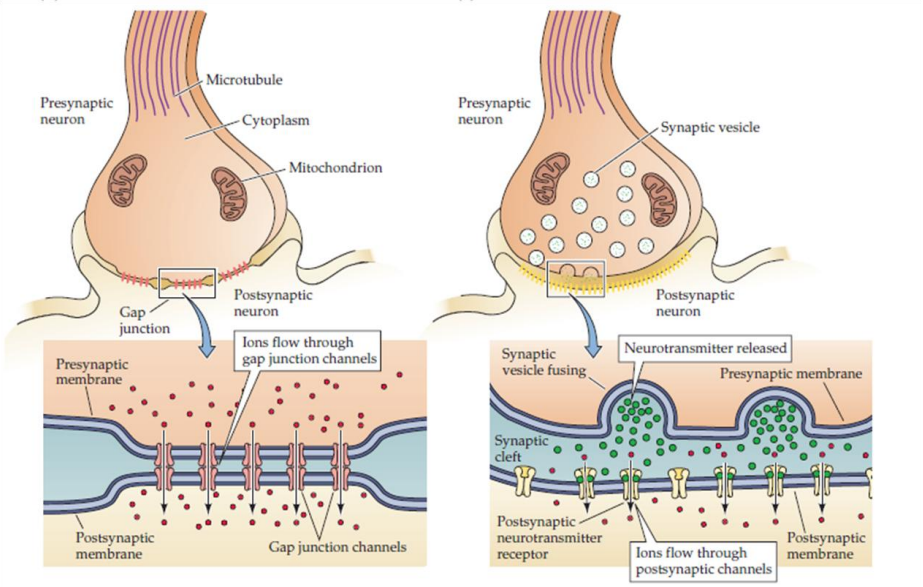


Figure 3 – Electrical and chemical synapses [5].

1.2.1. Chemical synapses/Neurotransmitters

Chemical synapses facilitate cell-to-cell communication, through the secretions of neurotransmitters, chemical agents released by the presynaptic neurons that produce secondary current flow in postsynaptic neurons by triggering specific receptor molecules. These synapses are activated through action potentials. The number of existing neurotransmitters is not known, but it is well over 100. Almost all neurotransmitters suffer a similar cycle: synthesis and casing into synaptic vesicles, release from the presynaptic cell, binding to postsynaptic receptors, and finally, removal and/or degradation. The release of the neurotransmitters is mediated by Ca^{2+} but the mechanism is yet to be fully understood. Many synapses release more than one type of neurotransmitter, and multiple transmitters can even be packaged within the same synaptic vesicle [5].

1.2.2. Electrical synapses

Activation of these synapses generates synaptic potentials, which permit transmission of information from one neuron to another. Synaptic potentials serve as the means of exchanging information in complex neural circuits in both the central and peripheral nervous systems [5]. Electrical synapses occur at all levels of the mammalian motor system [10].

1.2.3. Long term potentiation and synaptic plasticity

The strength of both forms of synaptic transmission can be improved or weakened by cellular activity. This synaptic plasticity in nerve cells is vital to memory and other higher brain functions like learning [1,2]. One of the most extensively studied candidate memory mechanisms is the synaptic phenomenon called long term potentiation (LTP) [1].

Long term potentiation of chemical synaptic transmission is one of the most extensively studied physiological models for memory formation in the mammalian brain. LTP is the consequence of coincident activity of pre and post-synaptic elements, resulting in a facilitation of chemical transmission that lasts for hours *in vitro* and can persist for periods of weeks or months *in vivo*. A large amount of evidences is now available demonstrating that LTP and memory are supported by similar molecular mechanism [11].

It is not yet possible to conclude definitely that LTP provides a mechanism for the neural basis of learning, but it is certainly a convincing physiological model for these processes. Animal studies during the last thirty years or so have covered a wide variety of preparations, from dissociated cell cultures to awake, freely moving animals. LTP is often induced in animals using repeated trains of high frequency stimulation, spaced at a frequency that mimics a spontaneous 5-7Hz neural rhythm. Nevertheless, only recently has progress been made in the study of LTP in humans. One of the techniques now in use is the transcranial magnetic stimulation (TMS) in which the cerebral cortex of an awake human subject can be stimulates non-invasively, with a remote hand-help apparatus [11].

1.3. The central and peripheral nervous systems

The nervous system can be divided into two main parts: the central nervous system (CNS), which consists of the brain and spinal cord, and the peripheral nervous system (PNS), composed of the cranial and spinal nerves, and associated ganglia [12]. As illustrated by the figure 4, the central nervous system is composed of the brain and the spinal cord, that lie within the bones of the skull and vertebral column [8]. The peripheral nervous system includes nerves, ganglia, and the enteric nervous system [1] (Figure 4).

CENTRAL NERVOUS SYSTEM	FUNCTION
Brain	Control centre
Spinal cord	Central relay centre
PERIPHERAL NERVOUS SYSTEM	**FUNCTION**
Somatic nervous system	
Sensory nerves	Transduction
Motor nerves	Carry motor commands
Autonomic nervous system	
Parasympathetic nervous system	Maintain homeostasis
Sympathetic nervous system	Stress response
Enteric nervous system	Digestion

Figure 4 – Divisions of the nervous system [adapted from 9].

The CNS (in focus on this project) is frequently considered to have seven parts, which correspond to the different segments of the embryonic CNS: the spinal cord, the medulla, the pons, the cerebellum, the midbrain, the diencephalon and the cerebral hemispheres. The diencephalon and the cerebral hemispheres are collectively called the forebrain. There are various subdivisions of the forebrain; the most evident anatomical structures are the prominent cerebral hemispheres. In humans, the cerebral hemispheres are proportionally larger than in any other mammal [5].

1.3.1. Prefrontal cortex

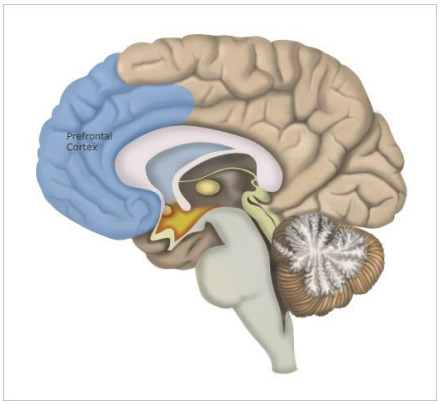


Figure 5 – Localization of the prefrontal cortex (in blue) [C].

The prefrontal cortex (PFC) consists of a collection of cortical areas that vary from one another on dimension, density, and distribution of their neurons and it is well positioned for a central role in cognitive control (the ability to take charge of one's actions and direct them toward the future) (Figure 5). It receives information from and sends projections to forebrain systems that process information about the external world, motor system structures that produce voluntary movement, systems that strengthen long-term memories, and systems that process information about affect and motivational state. An anatomy such as this suggests that the PFC may be important for composing external and internal information needed to produce complex behavior [1].

This area reaches the greatest relative size in the human brain and is thus thought to be the neural basis of the mental qualities that are considered intelligent. It takes up a far greater proportion of the human cerebral cortex than in other animals, which suggests that it might contribute to those puzzling cognitive capacities that separate humans from animals [1]. The prefrontal cortex is involved in the integration of cognitive and emotional information for attentional processing [13,14,15].

Some studies in rats, monkeys and humans agree that the prefrontal cortex contributes to executive functioning, i. e., the set of cognitive control processes that are necessary for optimal scheduling of complex sequenced behavior, including attentional selection and resistance to interference, decision-making, task switching, monitoring, behavioral inhibition and planning. The PFC is also strongly implicated in working memory processes. Working memory is a temporary memory system that comprises distinct but overlapping cognitive processes used for the active maintenance and elaboration of task-relevant information [16]. Also, deficits in working memory, associated with PFC dysfunction, are characteristic of several neuropsychiatric disorders, including depression, Parkinson's disease and schizophrenia [17,18,19,20]. In this view, the PFC acts as a note pad that sustains the inputs it receives from other cortical areas. Working memory maintenance functions are central to cognition. Complex, goal-directed behaviors typically are drawn out and thus are impossible without mechanisms that extend information over time [1].

There is plenty of evidence that these mechanisms are abundant in the PFC. This hypothesis has its basis in observations of deficits in performance of delayed response tasks following PFC damage and the fact that its neurons show sustained activity over a memory delay. Other deficits following PFC damage are thought to be consequences of the working memory deficit. For example, patients might be impaired at planning because they are unable to keep in mind all the different variables that need to be considered [1].

These studies in different species raise a very important question: because the volume of the cerebral cortex of rats is about a hundred times smaller than that of macaques and a thousand times smaller than that of humans [20], it is fair to say that the extent to which these processes can be considered functionally homologous in different species remains controversial. Even so, considering Rose and Woolsey's definition of prefrontal cortex [22] as cortex in receipt of reciprocal connections from the mediodorsal thalamus, as well as other criteria, several distinct regions of PFC can be identified in the rat. The rodent PFC is subdivided in three main areas: a ventrolateral area that regulates the control of socio-affective behaviors, a dorsomedial (prelimbic PL and cingulated Cg) area that is involved in working memory and a ventromedial area (IL) that plays an important role in visceromotor behaviors. The dorsomedial and the ventromedial areas are considered to represent the medial PFC, or mPFC. [23] (Figure 6)

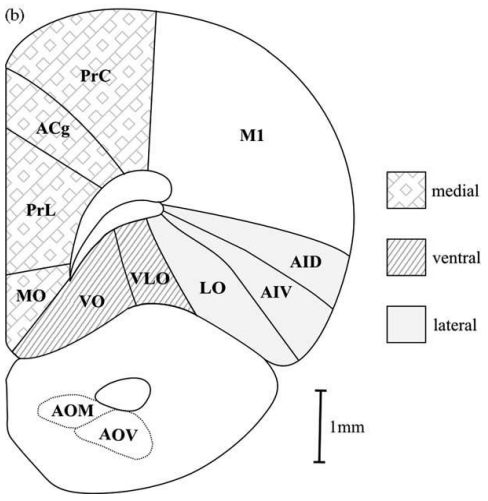


Figure 6 – Illustrative diagram of the rat PFC [adapted from 16].

According to Uylings, in rats, the medial prefrontal cortex (mPFC) is homologous to the dorsolateral PFC in primates. Definitely, the rat PFC is not as differentiated as it is in primates, but dorsolateral features, both anatomical and functional ones, are shown in rats [24].

The neurons of this region are capable of a correspondingly rapid modification of their properties to meet task demands. For example, monkeys can learn new conditional visuomotor associations in just a few trials and as they do, PFC neural activity rapidly modifies to reflect the new contingencies [1]. Furthermore, the PFC has extensive, mainly ipsilateral connections with other cortical areas, including the hippocampus [25].

1.3.2. Hippocampus

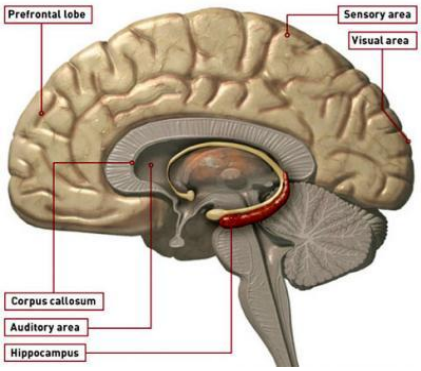


Figure 7 – Localization of the hippocampus (in red) [D].

The hippocampus is a bilateral incurved seahorse-shaped structure of the cerebral cortex [26], situated in the medial portion of the temporal lobe [5](Figure 7). It is one of the most extensively studied structures in the brain – the first findings in hippocampus using the Golgi method led to the discovery of structural plasticity in other brain regions [27]. The hippocampus is involved in declarative, episodic, contextual and spatial learning and memory, and it is also plays a role in the control of autonomic and vegetative functions. This structure

is vulnerable to damage by stroke and head trauma and susceptible to damage during aging and repeated stress [28].

The function of the hippocampus may vary between species. However, most findings from this area converge, both in the characterization of the kind of memory that is dependent on the hippocampal regions and the identification of functional domains as well as in the anatomical pathways associated with other types of memory. Some studies propose that primates and other mammals rely on the hippocampus to encode and consolidate memories of events and objects in space and time, in the same way as humans use this brain region for the initial encoding and consolidation of declarative memories [1].

The hippocampal memory system consists of the hippocampus proper, comprising the CA (*cornus ammon*) fields (CA1, CA2 and CA3), dentate gyrus (DG) and subiculum (Sub), and the entorhinal, perirhinal and postrhinal cortices in the adjacent region (Figure 8). The anatomy and circuitry of these regions, specially the hippocampus, are largely conserved across mammalian species [1].

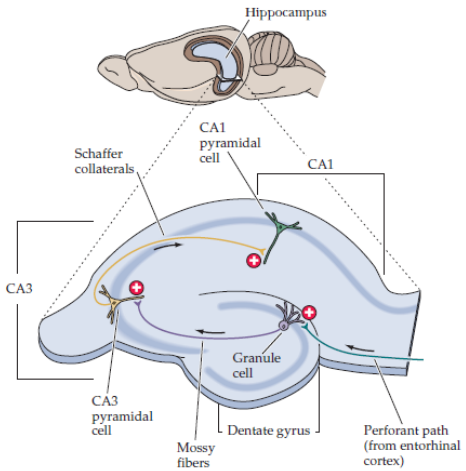


Figure 8 - Diagram of a section through the rodent hippocampus showing the major regions, excitatory pathways, and synaptic connections [5].

This hippocampal memory structures also depend on higher order regions in the cortex that are both the source of information to the parahippocampal region and hippocampus and the targets of projections originating from these regions. The parahippocampal region functions as a convergence spot for input from these cortical association areas and settles the distribution of cortical afferents to the hippocampus. These parahippocampal cortical areas are interconnected and send major efferents to the hippocampus. Within the hippocampus, an elaborate pattern of connectivity mediates a large network of associations, and these connections support forms of long term potentiation that could participate in the rapid coding of novel conjunctions of information [1] (LTP of mammalian excitatory synapses has been the most meticulously studied [5]). The outcomes of hippocampal processing are directed back to the adjacent cortical areas in the parahippocampal region, and the outputs of that region are directed back to the same areas that were the source of these inputs [1].

The hippocampus is connected with the PFC; this connection is made through axons, which originate in the subiculum and ventral CA1 subfields and terminate in the pyramidal cells and interneurons of the medial PFC [29,30].

Damage to the hippocampus and parahippocampal region produces anterograde amnesia, a memory impairment characterized by an incapability to make lasting memories of one's daily experience. That is, the hippocampal memory system usually supports remembering of new facts and events and makes this information available for conscious recollection afterwards. This

capacity is called declarative memory. Also, damage to these regions results in loss of memory acquired for some period before the damage, which is referred to as retrograde amnesia [1].

Patients with damage to the hippocampus are impaired on tests requiring them to inspect a scene with many objects and then to recall the locations of individual objects. The hippocampus has a role on both spatial and nonspatial memory. In rats, the hippocampus is essential not only for remembering locations but also for remembering odors and for learning associations between stimuli and rewards. In humans, likewise, memories of many kinds depend on this brain region [1]. Furthermore, it has been reported that patients that present cognitive impairments or depression present a considerable shrinkage of the hippocampus [28].

1.4. Stress

Stress is an aspect of our daily lives and conversations, and still there is considerable ambiguity in the meaning of this word [28]. This could be explained by the fact that it probably has a relatively different meaning for different people: physicians, psychotherapists, physiologists, and even you and me. According to Fink, it is a real or perceived challenge, either endogenous or exogenous, that perturbs the organism's equilibrium – homeostasis [31]. Humans and other animals respond to exposure to a determined stressor by mobilizing a host of neural, endocrine, neuroendocrine and metabolic systems [31].

Stress is frequently defined as a threat, real or implied, to homeostasis. In common usage, stress often refers to an event or succession of events that cause a response, usually in the form of “distress” but also, in a few cases, referring to a challenge that leads to a state of exhilaration, as in “good” stress. It is sometimes used in the negative way of “distress” and it is frequently used to describe a chronic state of imbalance in the response to stress [28].

The impact of stress on the brain has received much attention from both the neuroscience and lay communities [25]. In fact, the brain is the key organ for stress processes - it determines what individuals experience as stressful, it coordinates how individuals will deal with stressful experiences, and it changes both functionally and structurally as a result of stressful experiences [32]. We now know that the brain changes in structure and function with experiences, including those of chronic stress, that these changes in the brain represent “adaptive plasticity”, and that they are largely reversible and specific for the conditions that caused them. Quoting McEwen [33], “resilience in both brain structure and behavior is the name of the game in adapting to changing environments”.

There are two key aspects of the stress response. On one hand, the body responds to many experiences by releasing chemical mediators such as hormones, which promote a healthy adaptation to an acute stressor. On the other hand, chronic elevation of these mediators can cause pathophysiological changes. The importance of recognizing these two sides to the stress response,

the protective as well as the potential damaging effect, has led to the introduction of two terms: allostasis, which refers to the process of maintaining homeostasis by active means and allostatic load, meaning the wear and tear on the body and brain caused by use of allostasis, specially when this mechanisms is not turned off when stress is over or when it is turned on without the presence of a stressor [34,35].

Hormones play a critical role on the development and expression of multiple behaviors. Of all the endocrine axes, the hypothalamus-pituitary-adrenal axis (HPA) has been the most studied in the last years. It plays a fundamental role in the response to internal and external stimuli, including stressors. Abnormalities in the function of the HPA axis have been reported in patients that experience psychiatric disturbances. The activity of this axis is regulated by the secretion of corticotrophin (HLC) and vasopressin (AVP) by the hypothalamus, which activates the secretion of adrenocorticotrophin (ACTH), which finally stimulates the secretion of glucocorticoids by the adrenal cortex. The glucocorticoids are responsible for the negative feedback of the secretion of ACTH by the pituitary and of the HLC by the hypothalamus [36].

The adrenal gland is the source of the principal corticosteroids, namely cortisol, corticosterone and aldosterone. Like all other steroid hormones, these small molecules derive from cholesterol and pregnenolone, following a series of enzymatic conversions. It is important to note that cortisol is the principal

adrenocortical hormone found in humans and most other mammals, and that corticosterone is the predominant adrenocorticoid found in rodents and lagomorphs [37].

The brain appears to handle repeated stress over weeks by showing adaptive plasticity in which local neurotransmitters, as well as systemic hormones interact to produce structural and functional changes. Indeed, systemic levels of adrenal steroids and catecholamines, the classic stress hormones, do not tell the whole story as far as how the brain adapts [28].

Several forms of stress originate a generalized set of neuroendocrine and autonomic responses that help to mobilize and redistribute bodily resources, to facilitate coping with threats to homeostasis [15]

Stress is a major contributor to the etiology, pathophysiology and treatment outcome of mood and affective disorders; in addition, corticosteroids released in response to stress have been casually linked to psychopathology [38].

Chronic stress inflicts deep behavioral changes in humans and rodents, manifested as depressive-like symptoms, a hyperanxious state, and learning/memory deficits [39.28], along with structural damage [40] and impaired synaptic plasticity [41.42].

Damages of stress on the prefrontal cortex are well described and some studies are worth noting. Radley [15] stated that 6 weeks of repeated restrain

stress produced considerable decreases in apical dendritic length and branch number of pyramidal neurons. Both chronic stress and disturbances in the homeostatic mechanisms that regulate corticosteroid secretion are known to affect the structure and function of the rat mPFC: the structural changes include volumetric reductions in the most superficial layers and dendritic remodeling of pyramidal cells located in layer II/III of the mPFC [15,43,44].Another consequence of chronic stress on the PFC is the altered processing of input coming from the hippocampus [45].

In the rat hippocampus, stress has been shown to adversely affect neuronal metabolism, cell survival, physiological functions and neuronal morphology. Consequentially, some hippocampal functions, such as learning and memory are susceptible to disruption by stress. Recently, stress has also been found to impair hippocampus-dependent object-recognition memory. Furthermore, stress and corticosterone alter hippocampal dendritic morphology, and inhibit neurogenesis in the adult hippocampus [40].

Finally, and most importantly, Cerqueira [46] showed that the synaptic plasticity between the hippocampus and the prefrontal cortex, is diminished after stress and that the effects of stress on this pathway are not due to neuronal loss but to dendritic atrophy or retraction. Following that rationale, the next step is to understand how these effects can be reverted. For that reason, the present thesis intends to assess how and if a cognitive task that stimulates the hippocampus can or cannot revert that impairment of synaptic plasticity and

dendritic structures. Thus, we established that we would use the rat (Wistar Han strain) as the animal model. The line of work is essentially to subject the animals to a stress protocol followed by a training in the hole board task (HB) that stimulates the hippocampus. After that, using an electrophysiology protocol and neuronal reconstruction software, we will evaluate whether this training is enough to revert the loss of synaptic plasticity and the dendritic atrophy of the neurons due to stress.

2 | Goals

The main goal of this thesis was to evaluate the impact of cognitive enrichment (training in a cognitive task) in the reversion of stress-induced cognitive deficits. We decided to use an animal model (Wistar Han rat) to better explore the impact of our training procedure in two areas of the brain that are deeply affected by stress: prefrontal cortex and hippocampus.

In detail, the core objectives of this project were:

- to assess the synaptic plasticity between the PFC and the hippocampus on control and stressed animals and
- to correlate these synaptic alterations to the morphological alterations by analyzing the integrity of the neurons of these two brain areas.

3 | Methods

3.1. Animals and treatments

Two months old Wistar Han male rats (Charles River Laboratories, Barcelona, Spain) were housed in groups of 2 per cage, under the following conditions: *ad libitum* access to water and food, temperature at 20-24ºC, relative humidity of 55±10% and lights from 8 A.M. to 8 P.M. (Figure 9).

Experiments were conducted in accordance with local regulations (European Union Directive 86/609/ECC) and National Institutes of Health guidelines on animal care and experimentation.

Figure 9 – 2 months old Wistar Han rats on their cage, at the animal facility of the ICVS.

3.2. Stress protocol

The stress protocol chosen for this experiment was the Chronic Unpredictable Stress (CUS), adapted from Sousa [40]. This stress paradigm was shown to result in a pattern of stress-induced consequences, characterized by elevated plasma levels of corticosterone, and alterations in postmortem thymus weights. Also, the same study

showed that chronic stress severely disrupts two key processes attributed to the PFC: working memory and behavioral flexibility [46]. Furthermore, this protocol better mimics unexpected stressful life events encountered by humans, and since the type and time of each stressor are random, to avoid adaptation to the stressors [47]. Throughout the experimental period, this protocol was applied to 33 animals.

The protocol consisted on applying one of several stressors per day, during 28 days, in a random order and at random periods of the day. The stressors included:

- Overcrowding – This stressor consisted in placing 6 to 8 animals in a cage that would normally house 2, and so the animals had little space to move. It was applied for 1 hour;
- Restraint – To perform this, 2 animals were positioned in a plastic box with breathing holes, having practically no space to move and also no water or food. This stressor was also applied for 1 hour;
- Shaking – The animals were placed in a big plastic bag (usually 8 at a time) and shaken repeatedly during 15 to 20 minutes.
- Hot dryer – The animals remained in their cages, but the plastic lids were removed, leaving only the metal grid which holds food and water. Using a common hair dryer, and for 15 to 20 minutes, a hot air stream was pointed at the animals (repeating 10 seconds periods per cage).

- Cold Water – A 60 centimeter high plastic bucket was filled with cold water (tap temperature) up until 5 centimeters; the animals were then placed in the bucket for 1 hour. After this period they were towel dried and moved back to their cages.

3.3. Hole Board protocol

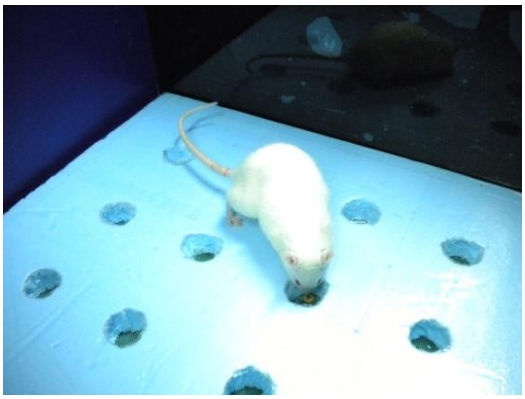


Figure 10 – Rat in the hole board apparatus, finding a reward.

The hippocampal formation is of crucial importance for cognitive processes and alterations in this brain area are associated with a selective impairment of hippocampus-mediated memory performance [48]. For this reason, the hole board food-retrieval task was chosen for this experiment, since it permits the assessment of working memory (WM) and reference memory (RM) [49,50].

The hole board apparatus consisted of a square board, 70x70cm, with 16 holes of 3,5cm diameter and opaque walls, as shown in Figure 10 (originally described by Ohl *et al.*, 2000[48]; adapted as in Depoortère *et al.* 2010[51]).

The rewards, C*heerio*®-like cereals, were placed in four of the holes; the disposition of the rewards was the same for each animal throughout the trials. To prevent the animals from using the scent of the rewards to find them, a few cereals were ground and scattered over the rest of the holes.

Two days before the beginning of the task, the animals entered a regimen of food deprivation (F.D.). In other words, the food was no longer available *ad libitum* - the rats could only eat for one hour per day. This allowed the animals to be hungry enough to perform the task; otherwise they would not have the necessary motivation to find the rewards. This F.D. continued during the 5 days of the task. One day before the task, a reward cereal was given to the animals so that they could get used to its smell and taste.

This task was carried out for a period of five days, with one trial per day.

At the beginning of each trial, the rat was placed on the center of the platform and allowed to explore. The trial ended whenever the animal found the four rewards, or after a maximum of 30 minutes. The number of right answers (nose poke on the holes that contained rewards), wrong answers (nose poke on empty holes), eaten rewards, repetitions after reward (nose poke on correct holes that no longer had the reward), time to find the first reward and the total time of the trial were registered, for each animal and each trial. The task was performed with 34 animals, 16 control and 18 stressed.

3.4. Electrophysiology

According to Squire *et al* [1], the LTP phenomenon is defined as a persistent increase in synaptic strength that can be induced rapidly by a brief burst of activity in the presynaptic neurons. These authors also state that the experimental interest of LTP can

be justified by the working hypothesis that this form of synaptic plasticity may participate in information storage in several brain regions.

It is also known that the exposure to acute stress is known to impair hippocampal LTP in rats [52], so by inducing LTP on a rat's brain and measuring the difference between the initial response and the one after the LTP, we can evaluate the synaptic plasticity of a certain area or pathway.

In this experiment, we intended to measure synaptic plasticity, specifically on the connection between the hippocampus and the prefrontal cortex (PFC). To do that, we decided to employ an electrophysiological protocol described by Cerqueira [46], that consists of three main steps: first, electrical stimulation of the CA1 pyramidal cells of the hippocampus while recording and measuring the response of the PFC to that stimulus; second, induction of Long Term Potentiation (LTP) by over stimulating the synapse, and theoretically increasing the PFC's response to an equivalent stimulus; and last but not least, a new measure of the response obtained on the PFC with the initial stimulus. These steps take respectively 30, 10 and 90 minutes (Figure 11).

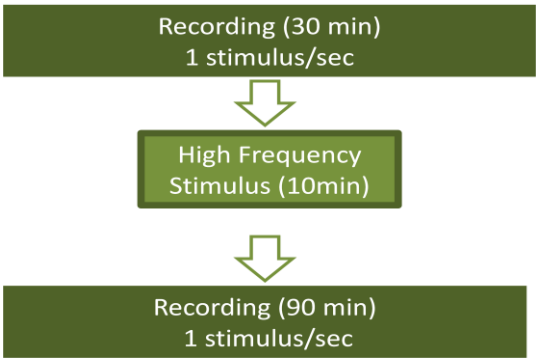


Figure 11 – Scheme of the 3 main steps of the electrophysiological protocol.

Experimental procedures for implantation and recording extracellular field potentials in the prelimbic area (PL) of the PFC were performed as described previously [53].

The animals were anesthetized at the animal facility, with an intraperitoneal (IP) injection of sodium pentobarbital stock solution 20% (Sanofi-Aventis, France) – average dosage of 0,025mL/100g of body weight. As they became unconscious, the animals were then taken to the electrophisyology room and, about 40 minutes after the first injection, received a second injection with half of the dosage of the first administration, diluted in 4/5 of saline. Throughout the experiment, the anesthesia was supplemented each 40 to 60mins with the same dosage.

Rectal temperature is maintained at 37°C by a homeothermic warming blanket (Stoelting, Ireland).

Once the rats were deeply anesthetized, they were placed in a David Kopf® stereotaxic frame (figure 12). The scalp was cut, exposing the skull. After identifying the Lambda and Bregma points, 2 small holes were drilled with a 1.6mm Ø electrical drill for the electrodes to be placed in the PFC and the hippocampus (Figure 13), and a third hole was drilled manually, on the cerebellum region. This third hole was used to place a bolt that acted as an active reference electrode to the other electrodes. The received signals resulted from the difference

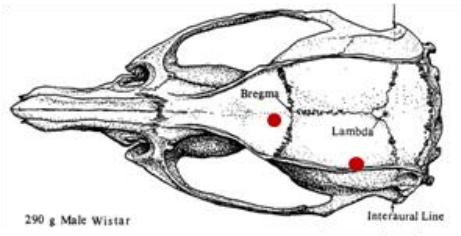


Figure 13 – Scheme of the rat's skull, showing the bregma and lambda points and the approximate localization of the electrodes positions [adapted from 52]

"PFC electrode-bolt" and "hippocampus electrode-bolt". The bolt was connected to the acquisition system through a nickel-chromium wire (Science Products, Germany). Another section of this wire was sewn onto the animal's neck muscle and it functioned as the ground reference, to electrically match the acquisition system and the animal's body.

A platinum/iridium recording electrode (Science Products, Germany) was placed in the prelimbic area (coordinates: 3.3mm anterior to Bregma, 0.8mm lateral to the midline, 4.0mm below Bregma) and a concentric bipolar tungsten/stainless steel electrode (WPI, USA) was positioned into the ipsilateral CA1 region of the ventral hippocampus (coordinates: 6.5mm posterior to Bregma, 5.5mm lateral to the midline and 5.3mm below Bregma), according to The Rat Brain in Stereotaxic Coordinates of Paxinos and Watson, 2005 [54].

Local field potentials obtained from both electrodes were amplified, filtered (0.1-3000Hz, LP511 Grass Amplifier, Astro-med, Germany), acquired and converted to digital information (Micro 1401 kmII, CED, UK) and recorded on a personal computer running Signal software (CED, UK).

The electrode inserted in the CA1 region was then used to deliver stimuli to induce a characteristic monosynaptic field postsynaptic potential (PSP) in the mPFC (visible on the oscilloscope, Tektronic TDS 3012B, USA). Test pulses (100 ms) were delivered every 30 s at an intensity enough to evoke a potential about 70% of its maximum (250-500 μA; S88X Grass Stimulator, Astro-Med, Germany). The evoked potential to such

stimulation is likely to reflect summated PSPs. Basal responses were recorded during 30 mins and followed by LTP induction, which was obtained performing high-frequency stimulations (HFS), that consisted of two series of 10 trains (250HZ, 200 ms) at 0.1 HZ, 6 min apart, delivered at test intensity. The amplitude of responses was measured for an additional 90 mins, stimulating each 30 s with the same intensity as on the first 30 mins. PSP amplitudes were analyzed using Signal Software (CED, UK; sampling rate 10 kHz) and expressed as a percentage of change of the mean responses to basal stimulation before and after HFS.

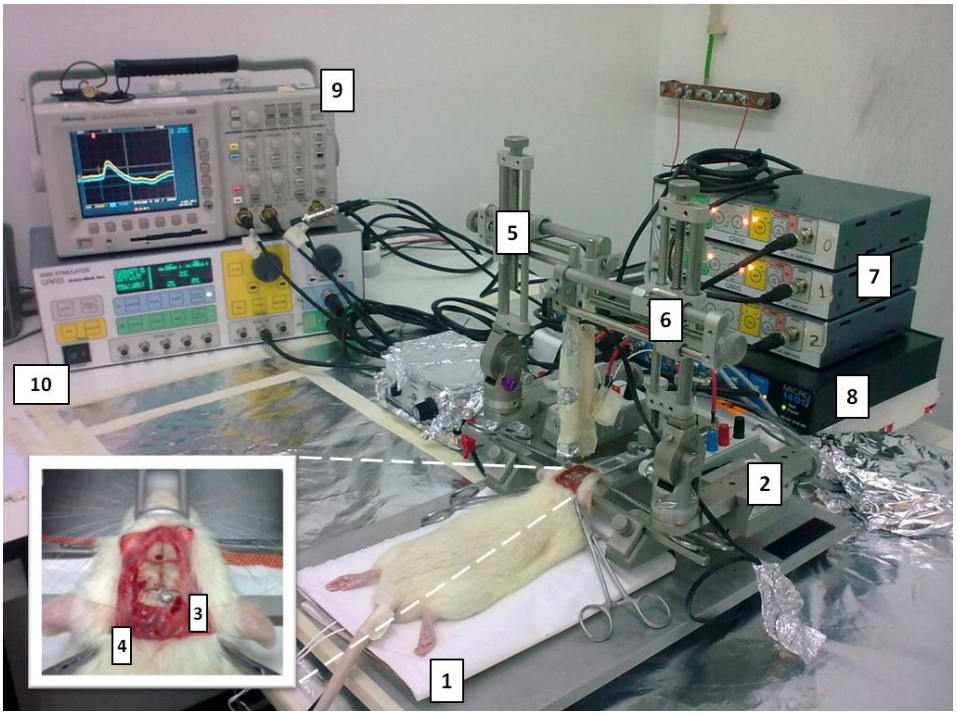


Figure 12– Rat in the stereotaxic frame and acquisition system apparatus. **1** – Homeothermic blanket; **2** – Stereotaxic frame; **3** – Bolt screwed to the skull on the cerebellum region; **4** – Nickel-chromium wire sewn to the muscle; **5** – Stereotaxic bar with PFC electrode; **6** – Stereotaxic bar with hippocampus electrode; **7** – Amplifiers; **8** – Analogue to digital converter; **9** – Oscilloscope; **10** – Stimulator.

After the electrophysiological protocol, a biphasic 1mA stimulus was delivered to both electrodes. The animals were sacrificed with a guillotine for rodents (Kent Scientific

Corporation, USA) and the brains were carefully removed and stored for two days in paraformaldehyde (PFA) 4%, after which they were sectioned in 50 pm slices on a freezing microtome and mounted on glass slides. The slices were then examined under a Stereoscope (Leica®, Germany). The lesion caused by the biphasic stimulus identified the electrode's position (see Annex 1).

In order to evaluate the synaptic plasticity of each animal, the response of the PFC to an electrical stimulus on the hippocampus was recorded and analyzed with Signal software. The analysis consisted of comparing the amplitude of the response before and after the induction of LTP. The figure 14 is a representation of the output given by Signal. The small vertical line in blue represents the stimulus and the bell-shaped curve represents the PFC's response, throughout time (s). A *script* file was created and we ran it through the data. This *script* gave a more reliable analysis of the data, since it automatically measured the amplitude of the response (represented by the red line) during the first 30 mins and during the final 90 mins, after the LTP. The difference between the two amplitudes is called potentiation, which is the 'growth' of the signal caused by the LTP. These potentiation values were compared between the groups and allowed to reach some conclusions about the synaptic plasticity of the different groups. The protocol was applied to a total of 49 animals: 16 controls, 10 controls that performed the HB task, 11 stressed and 12 stressed animals that performed the HB.

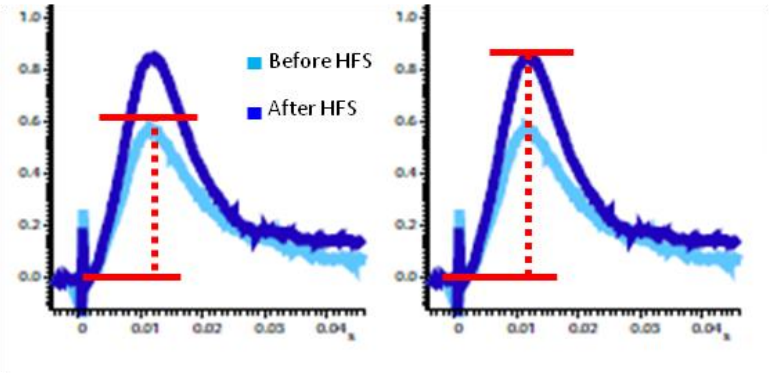


Figure 14 – Representation of the response of the PFC to an electrical stimulus. Blue lines represent the signal and the red lines and dots represent the amplitude of the signal.

3.5. Morphology Analysis

For this part of the experiment, we intended to evaluate the morphological state of the neurons of the animals and compare it between groups. For that we decided to use a 3D reconstruction technique that allows assessing the morphology of the neurons (number of dendrites, branches, spines, among others).

The actual structure of the neuron did not become understandable until Ramón y Cajal, in the late nineteenth century, began to use a silver staining method proposed by Golgi. This method has two major advantages. The first is that the silver solution stains only 1% of the cells in any region of the brain. The reason for this is not yet fully understood, but it allows the study of single neurons in isolation from the neurons of the vicinity. The second advantage is that the stained neurons are outlined in their entirety: cell body, axon and complete dendritic tree [2]. The Golgi solution is based on the reaction off chromates salts with heavy metals, usually silver or mercury [56].

Before the staining process, it is necessary to remove all of the blood from the brain, since it would interfere with the staining and the visualization of the neurons. For

this reason, the animals were perfused transcardially. First of all, they were deeply anesthetized with sodium pentobarbital (0.3-0.5 mL per 100g of body weight) and perfused with saline solution (0.9%). The perfusion consisted in opening the rat's abdomen using a scalpel, cutting through the peritoneum, the diaphragm and reaching the heart. A needle connected to a peristaltic pump (Gilson, Inc; USA) was inserted on the left ventricle and a cut was made on the right atrium. This allowed the saline solution to replace the blood all over the animal body, and the blood to exit through the right atrium. After approximately 15 to 20mins, all the blood was replaced by the saline solution. The brains were removed and stored in Golgi solution for 14 days. After that period, the Golgi solution was removed and replaced by a saccharose solution (30%) for two days. The rat's brains were then sectioned in 80pm slices and mounted on glass slides.

Afterwards, using the Neurolucida software (MBF Bioscience, USA), the brain slices were analyzed. When used in connection with a light microscope, this software utilizes a computer controlled and motorized XYZ stage for integrated steering through tissue sections. It provides tools for visualizing a captured image and tracing neuronal branches, outputting 3D coordinates and connectivity of trace points. When the tracing was complete, the final product was a digitized, morphologically realistic neuronal representation that could be transferred to NeuroExplorer (MBF Bioscience, USA), a 3D visualization and morphometric analysis program that permits the determination of total dendritic length and number of spines per neuron [6,56].

In the PFC, pyramidal neurons from the II and III layers of the PL were reconstructed and the spines of specific segments were identified and counted on both the apical and basal dendrites (Table 1):

- Apical dendrite - 3 segments at a distance of 50-100μm from the cell body (basal apical dendrite) and 3 others at 150-200μm (distal apical dendrite), randomly selected (see figure in annex).
- Basal dendrites – in 3 segments at 50-100μm from the cell body, also randomly selected.

In the hippocampus, granular neurons of the dentate gyrus (DG) (hippocampus) were reconstructed, and the spines of specific segments were identified and counted on the basal dendrites:

- Basal dendrites – in 3 segments at 50-100μm from the cell body, also randomly selected.

After the reconstructions, and using the NeuroExplorer Software, the following data were collected:

The morphological analyses were performed at the Champalimaud Center for the Unknown, Algés. This reconstruction was performed in 18 animals (4 controls, 4 stressed, 6 stressed plus HB and 4 control plus HB).

PFC	Basal dendrites								
	Dendrites				Spines				
	Number of dendrites	Number of branches	Number of ends	Length	Thin	Mushroom	Thick	Ramified	Density
	Apical dendrites								
	Dendrites				Spines				
	Number of dendrites	Number of branches	Number of ends	Length	Thin	Mushroom	Thick	Ramified	Density
Hip	Apical dendrites								
	Dendrites				Spines				
	Number of dendrites	Number of branches	Number of ends	Length	Thin	Mushroom	Thick	Ramified	Density

Table 1- Collected data from 3D reconstruction of PFC and hippocampal neurons.

3.6. Statistics

After the data have been collected, statistical analysis was performed. The normality of the values was tested with Kolmogorov-Smirnov. The differences between groups were analyzed with the one-way ANOVA or repeated measures ANOVA, whenever the data complied with the assumptions of normality of the distribution and if the variances were homogenous. Individual differences between groups were analyzed post-hoc with Tukey's honestly significant differences test. Significant differences were considered for $p<0,05$. When present, the outliers were removed. The statistic analysis was performed with SPSS Statistics 2009 software (IBM, USA). The graphics were designed with Microsoft Excel 2007 (Microsoft, Inc.; USA) and GraphPad Prism 5 (GraphPad Softwares, Inc; USA).

4 | Results

4.1. – Hole Board

The hole board task (HB) is designed to asses an animal's spatial reference and working memory abilities, by showing a reduction in the number of wrong answers and of repetitions after reward, respectively. Additionally, other parameters assessed are the number of correct answers, the number of rewards eaten, the latency to find the first reward and the total time of the trial. All these results will be presented as an average performance of each group.

An example of an individual performance from a control animal is shown on the figure 15. Note the decrease in the number of wrong answers and the number of entries into holes where a reward had been eaten, demonstrating learning of the task, and good reference and working memory skills.

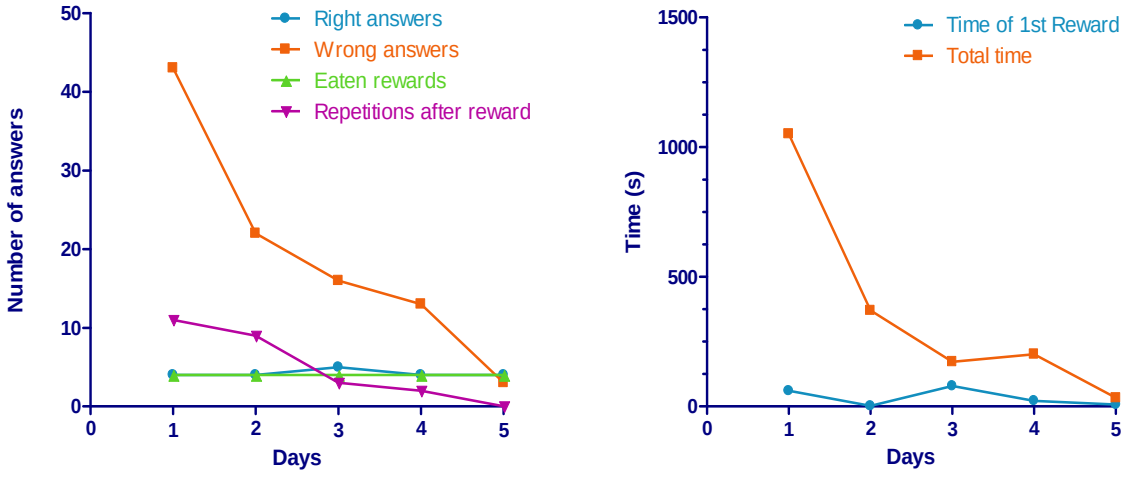


Figure 15– An example of an individual analysis of the performance on the Hole Board task in a control animal.

4.1.1. – Group results

Compared with controls, stressed animals had a tendency to give less correct responses in the first days of the task (reference memory, $F_{4,21}$=3,629 p=0,069), but this improved throughout testing until their performance was similar to the controls (figure 16). Interestingly, control animals also had a higher number of wrong answers in the first days of testing ($F_{4,21}$=17.453 p=0.000), which might be explained by an increased exploratory behavior, when compared to the stressed animals. Importantly, both groups of animals significantly decreased the number of wrong answers throughout the test ($F_{4,21}$=16.365 p=0.000) (figure 16).

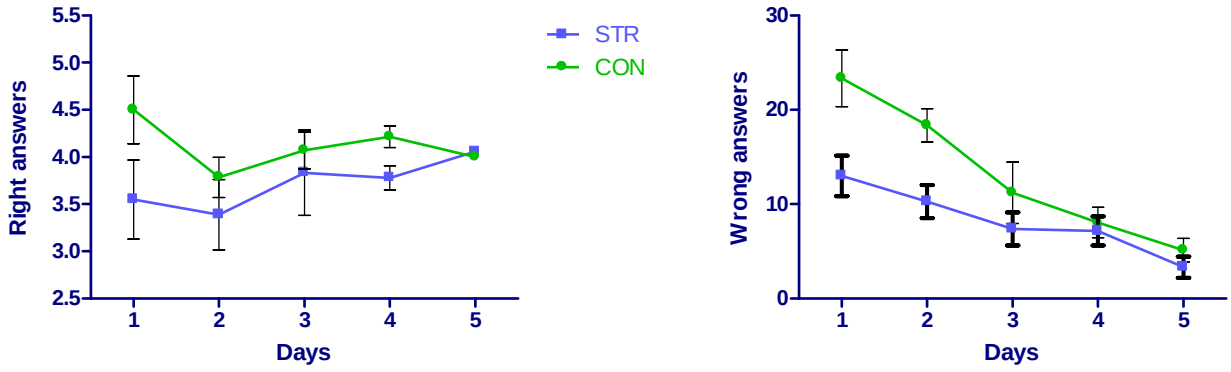


Figure 16 – Group analysis of the performance on the hole board task – number of right and wrong answers. The results represent mean ± SEM. N(CON)= 14, N(STR)= 12.

The increased exploratory behavior of control animals might also explain why this was the group displaying the most repetitions after reward in the first days ($F_{4,21}$=9.328 p=0.005), although in the last days there were no differences between groups (figure 17). The repetitions throughout the days decreased significantly ($F_{4,21}$=4.310 p=0.01). Importantly, although stressed animals ate only an average of 2 pellets per trial in the first day ($F_{4,21}$=7.103 p=0.014), all animals ate the 4 rewards in the last days of the experiment (figure 17).

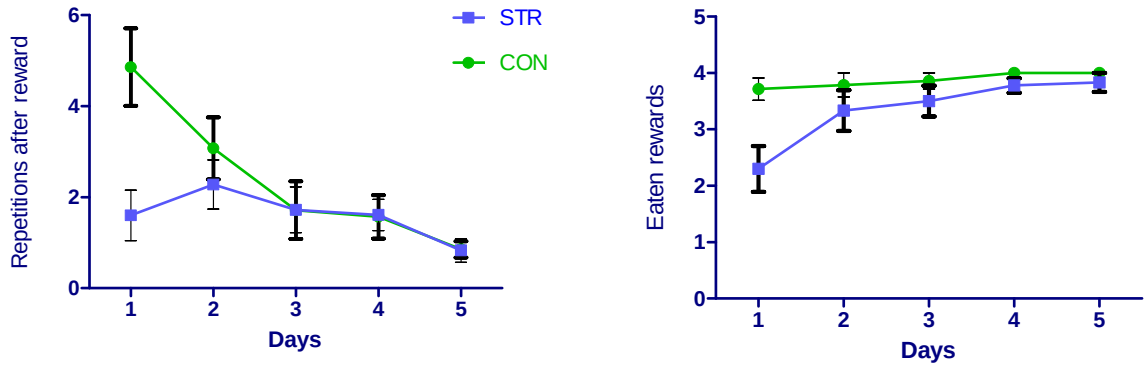


Figure 17 - Group analysis of the performance on the hole board task – Number of repetitions after reward and number of eaten rewards. The results represent mean ± SEM. N(CON)= 14, N(STR)= 12.

This can be explained by the lack of exploratory behavior on the stressed animals. Despite the fact that the time needed to find the first of the 4 rewards on the first 2 days was longer for stressed animals, both groups progressed to very close values after the 3rd day of testing $F_{4,21}$=4.046 ;p=0.056) (figure 18) Similarly, the time needed to find the 4 rewards and thus finishing the task was

also lower on the controls, when compared to the stressed animals, but again this group evolved to values close to the control animals (figure 18).

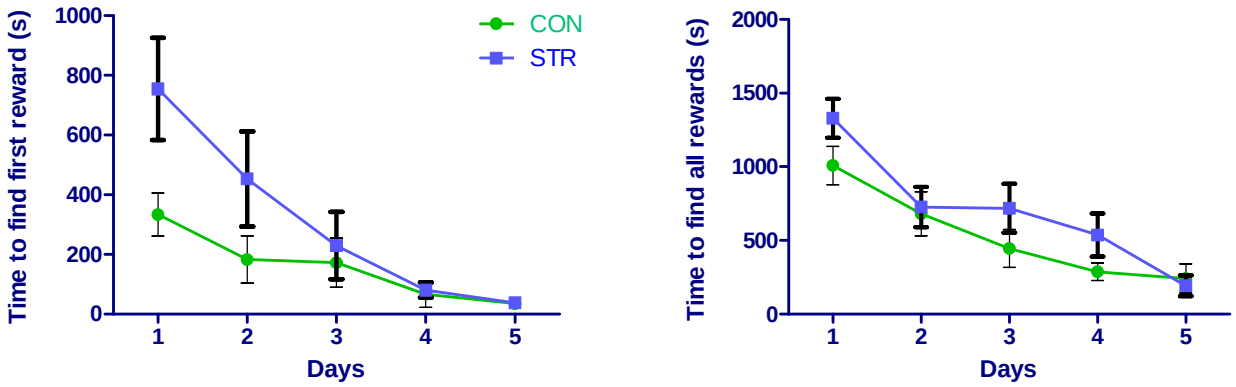


Figure 18 - Group analysis of the performance on the hole board task – Time to find the first reward and total time to finish the task. The results represent mean ± SEM. N(CON)= 14, N(STR)= 12.

4.2. – Electrophysiology

From the animals subjected to this protocol, not all could be used for this analysis, either because they died during the protocol (the reason may lie on the first bottle of anesthetic used not being stored on ideal conditions) or because the electrode failed to be positioned on the right coordinates (see annex – stereoscope pictures of brain slices, showing right and wrong positioning of the electrode).

The increase of the PFC's signal amplitude was used to assess the synaptic plasticity of the animals. The potentiation values were calculated comparing the amplitude of the PFC's signal before and after the high

frequency stimulus (HFS). Data were normalized and the results represent the potentiation of the signal in 3 time points after the HFS. These 3 time points were generated to have a better understanding of the potentiation throughout the 90 minutes after the HFS, if it was gradually increasing, decreasing or if it was constant. The results show that there were no differences between the 3 time points, suggesting that the potentiation remained constant.

The potentiation values varied between the groups. As expected, the stressed (STR) animals had lower potentiation than the controls (CON), indicating loss of synaptic plasticity. It is important to note that the stressed animals that performed the hole board task (SHB) had a higher potentiation than the STR, suggesting that the task might restore some synaptic plasticity in this specific circuit (4H).

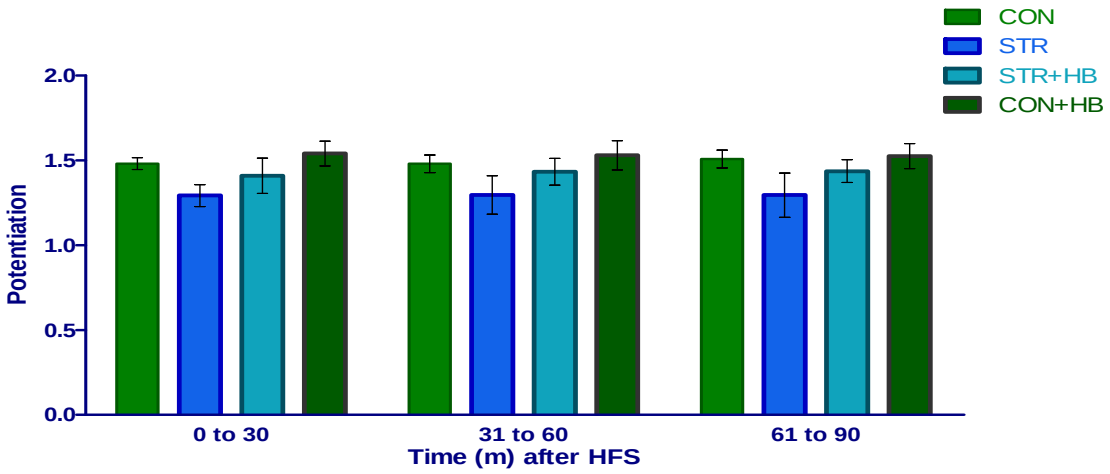


Figure 19– Analysis of the increase in the PFC's signal amplitude on the 4 studied groups: controls (CON), stressed (STR), stress+Hole board (STR+HB) and controls+hole board (CON+HB) The results represent mean ± SEM. N(CON)= 8, N(STR)= 6, N(SHB)= 7, N(CHB)= 8.

The group of control animals that performed the task (CHB) was used to assess if he task itself was stressful or if it would disturb the circuit between the PFC and the hippocampus, but since it had no significant differences when compared to the controls, it seems that the task has no negative influence on this experiment.

4.3. – Morphology analysis

The 3D reconstruction of neurons provided relevant information about the morphology of the neurons from each group of animals. There were reconstructed 5 neurons from each region and per animal.

In the beginning of this experiment we formed 4 groups of animals, but due to a problem during the Golgi impregnation, the slides of the control animals that performed the HB task were not usable. The brain slices were broken and too dry so it was impossible to find enough neurons for the analysis. For this reason and for lack of time to repeat this part of the work, the results will only feature 3 groups of animals: control (CON), stressed (STR) and stressed animals that performed the HB (SHB).

4.3.1. – Prefrontal Cortex neurons

4.3.1.1. – Basal dendrites

From the PFC region we reconstructed 5 pyramidal neurons per animal.

The number of basal dendrites on the STR animal's neurons it less than in the CON group. The SHB animals have a number of basal dendrites similar to the CON. The number of branches is higher on the CON when compared to STR animals and the SHB group showed an intermediate value between the two other groups. This difference between the CON and SHB groups is significant ($p=0.037$). The number of dendritic ends is also higher on controls, when compared to SHB and STR. In fact, the difference between the CON and SHB is significant ($p=0.023$) (figure 20).

Similarly, basal dendrites were shorter in the STR animals than in the CON; the SHB animal's basal dendrites were longer than the STR and shorter than the CON (figure 20).

Concerning the number of spines, although we have classified 4 types of spines, only two types were considered individually: thin and mushroom. The reason for this is that these types of spines are more common than the ramified and the thick spines. Nevertheless, the spines were all accounted for so that we could assess the spine density of the neurons. The number of these two types of spines was not the anticipated, since the 3 groups showed very similar

values. This can be due to differences on the impregnation with the Golgi solution, or even the quality of the solution itself, since it can influence the amount of visible spines. Even so, the dendritic density demonstrated, as expected, that basal dendrites of CON animals had higher spine density when compared to STR animals (the difference is significant: p=0.023) and that these animals had lower density than the SHB animals. The difference between SHB and CON animals was also statistically significant (p=0.002) (figure 20).

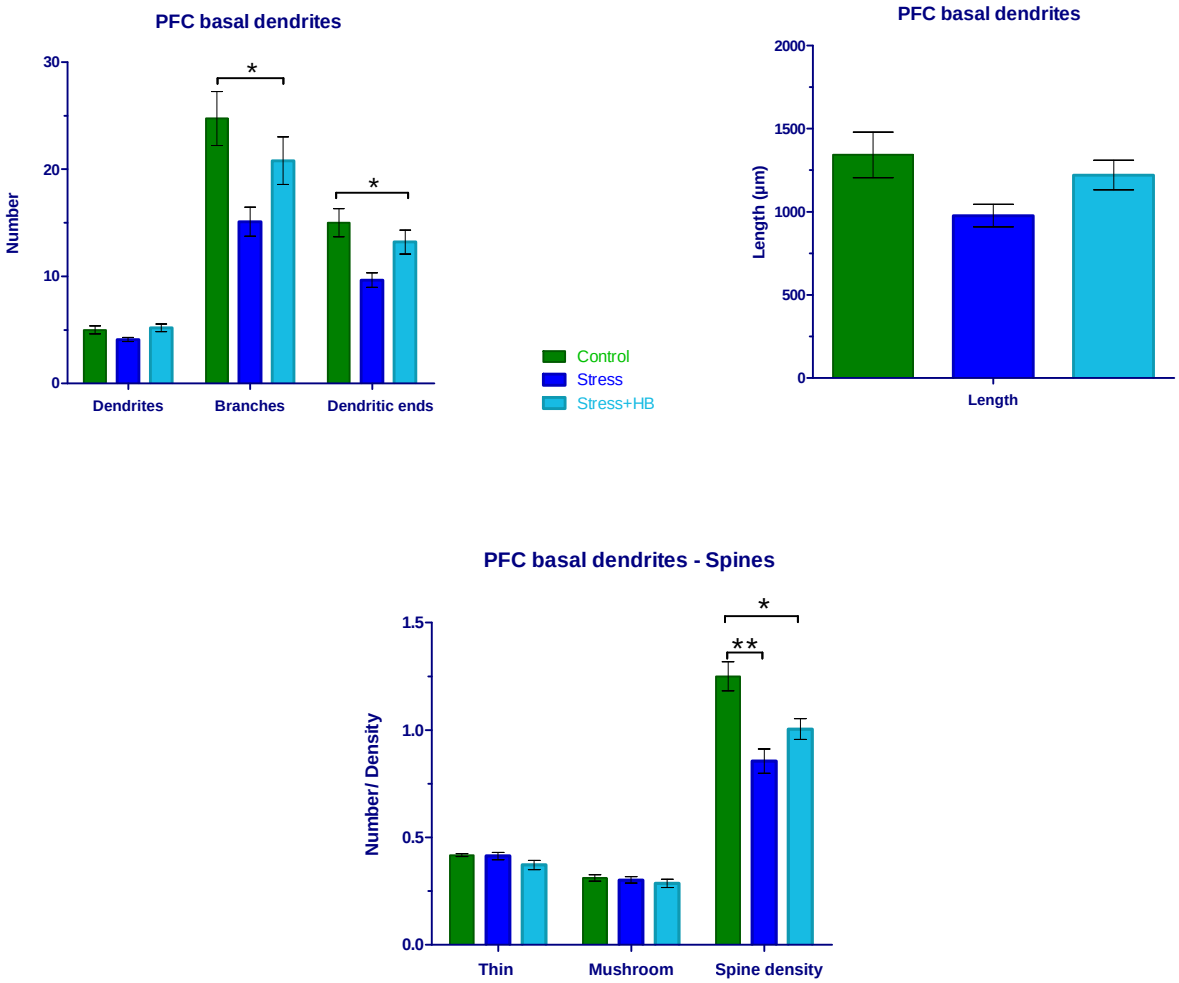


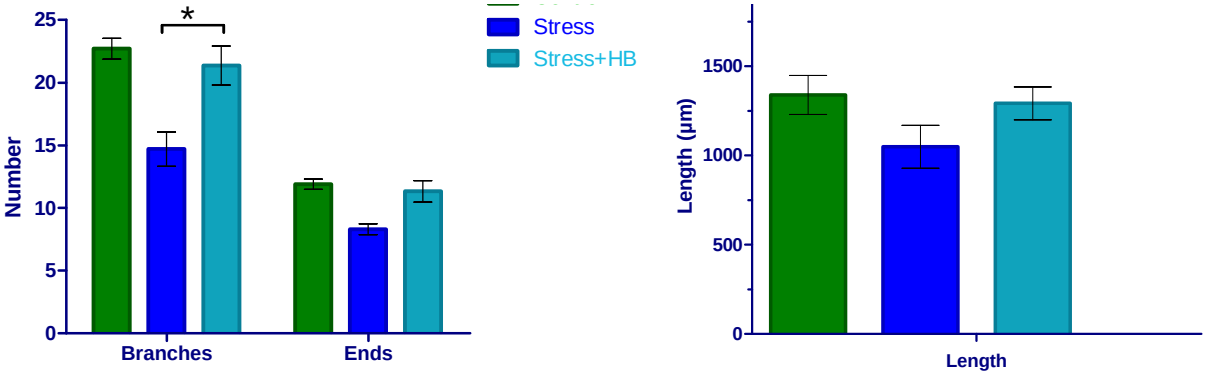
Figure 20 – Group analysis of the morphology of the PFC neurons: number of basal dendrites, branches and dendritic ends; length of the basal dendritic branches; number of thin and mushroom spines and spine density of the dendrites. The results represent mean ± SEM; *p<0.05 **p<0.001. N(CON)= 4, N(STR)=4, N(SHB)= 6

4.3.1.2 – Apical dendrites

The apical dendrites of the STR animals showed fewer branches and fewer ends than the CON's apical dendrites. There was a significant difference between these two groups (p=0.009). When comparing STR animals to SHB,, the latter featured a higher number of branches, but not as high as the CON animals. The differences between STR and SHB on this parameter were significant (p=0.016). The number of ends of the SHB animals was flanked by the CON and the STR values, being that the STR animas had the lowest number of ends (figure 21).

Lengthwise, the CON animals showed long apical dendrites, followed by the SHB and the STR animals, as expected (figure 21).

Figure 21 – Group analysis of the morphology of the PFC neurons; number of apical dendrites, branches and dendritic ends; length of the apical dendritic branches. The results represent mean ± SEM; *p<0.05 **p<0.001. N(CON)= 4, N(STR)=4, N(SHB)= 6



The spine number and density was assessed in two regions: proximal (50-100 µm away from the cell body) and distal (150-200 µm away from the cell body). The number of thin and mushroom spines did not provide helpful data on any of the two regions, probably for the same reasons that were explained for the basal dendrite's spines. On the other hand, the spine density parameter was useful: in both regions, the CON's apical dendrites had a higher spine density than the STR, and the SHB animals showed an intermediate value. On the proximal regions, the differences between CON and STR animals was significant (p=0.000), as well as the difference between CON and SHB (p=0.043) and between STR and SHB (p=0.003). Similarly, on the distal regions the difference between CON and STR, CON and SHB and STR and SHB were also significant (p=0.001; p=0.043 and p=0.001 respectively) (figure 22).

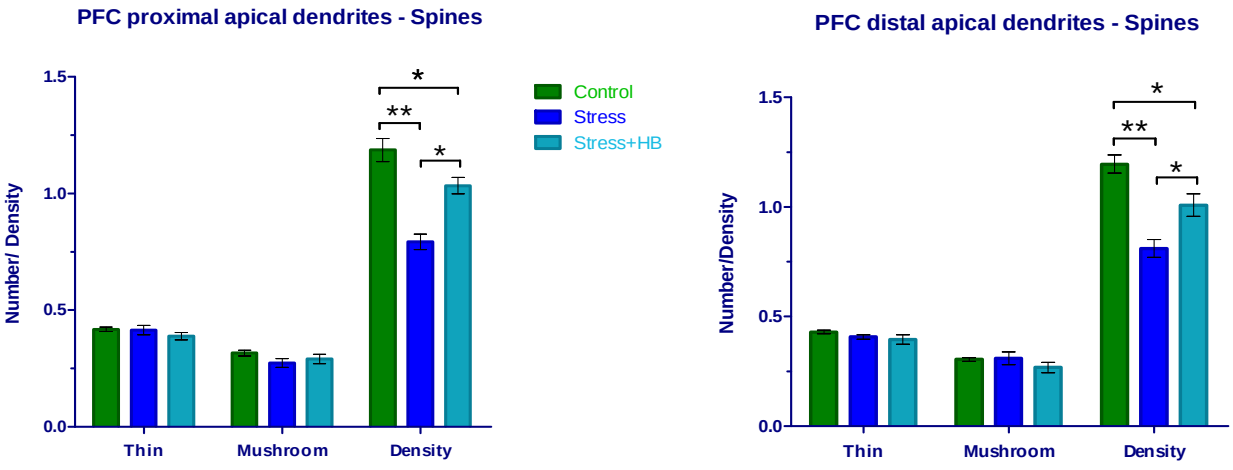


Figure 22 – Group analysis of the morphology of the PFC neurons: number of thin and mushroom spines and spine density on the proximal fragments (50 to 100µm from the cell body); number of thin and mushroom spines and spine density on the distal fragments (150 to 200µm from the cell body) of the dendrites. The results represent mean ± SEM; *p<0.05 **p<0.001. N(CON)= 4, N(STR)=4, N(SHB)= 6

4.3.2. – Hippocampal neurons

From the hippocampal region we also reconstructed 5 pyramidal neurons per animal.

Regarding the hippocampal neurons, the number of dendritic branches varied between the three groups, as anticipated. The SHB animals showed a higher number of branches when compared to STR animals and a lower number when compared with CON. The difference between the CON and STR animal's number of branches was significant (p=0.002) as well as the difference between STR and SHB animals (p=0.008). Comparing the number of ends, the same tendency is observed: higher number of dendritic ends on the CON animals, lower on the SHB and even lower on the STR animals. The differences between CON and SHB animals was significant, as was the differences between STR and SHB animals (p=0.002 and p=0.002) (figure 23).

The values for the length of the hippocampal dendrites were the expected: longer dendrites on the CON group, when compared to the other two groups; the SHB animals showed shorter dendrites than the CON but longer than the STR animals. Significant differences were observed between the CON and the SHB group (p=0.003) and between STR and SHB animals (p=0.006).Similarly to the analysis of the PFC's neurons, also in the hippocampal region the measurements for the thin and mushroom spines were not the expected. Nevertheless, the spine density showed parallel results to the previous analysis.

The CON animals evidenced a higher spine density when compared to STR animals, the last having lower density than the SHB group. The differences between CON and SHB groups were statistically significant (p=0.007) (figure 23).

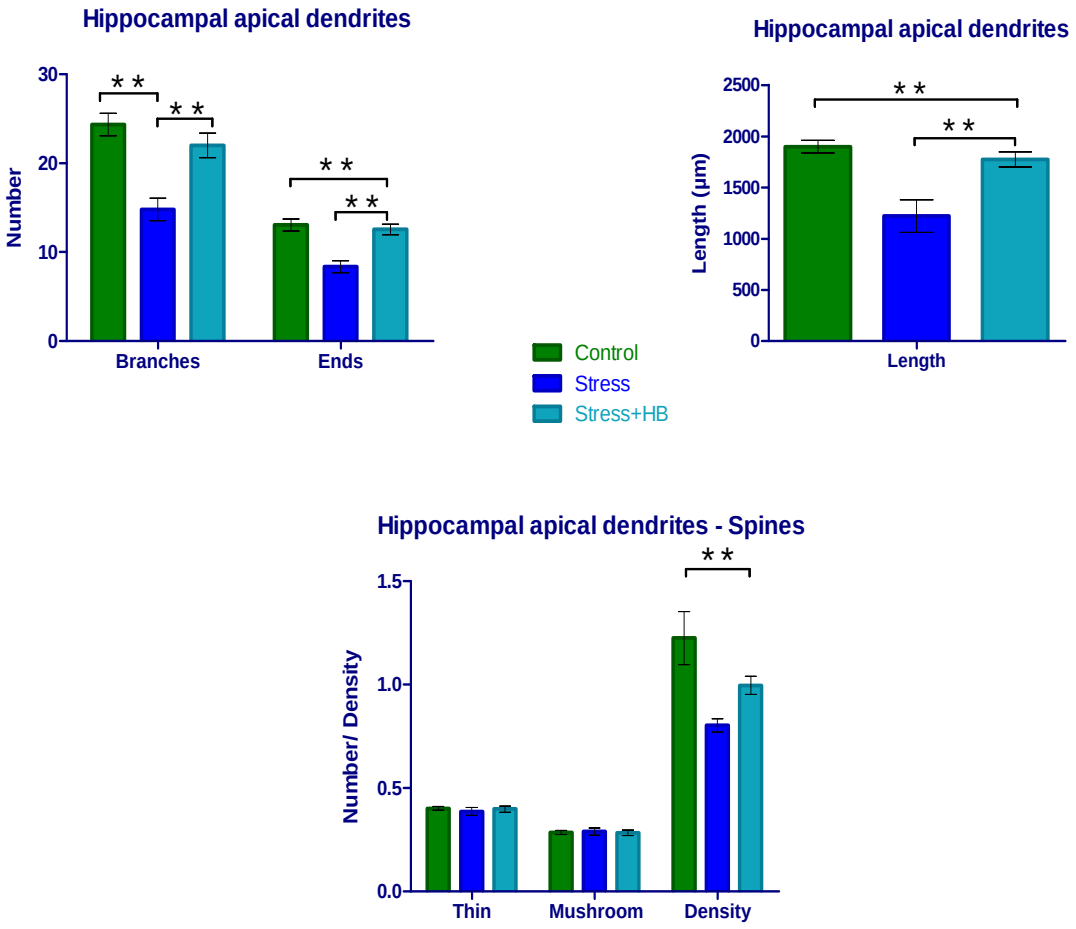


Figure 23 Group analysis of the morphology of the hippocampus neurons; number of apical dendrites, branches and dendritic ends; length of the apical dendritic branches; number of thin and mushroom spines and spine density of the dendrites. The results are represent by mean ± SEM; *p<0.05 **p<0.001. N(CON)= 4, N(STR)=4, N(SHB)= 6

5 | Discussion and conclusions

In the present work we showed that training in the hole-board, a reference memory task, can promote recovery from stress-induced cognitive deficits in the adult rat. Importantly, we also show that this recovery is task specific, since training improved performance in a different reference memory task but not in a behavioral flexibility task, both of which were shown to be altered upon chronic stress. By also exploring the underpinnings of such recovery, we further show that, when compared with chronically stress rats, trained animals displayed longer dendritic trees on their DG granule cells (but not on pyramidal cells of the PFC) and higher LTP levels inducible between the hippocampus and the prefrontal cortex.

5.1. Hole Board

Since chronic stress exposure is known to induce reference and working memory deficits, we predicted that STR animals would have more difficulty in completing the HB task. Indeed, as expected, STR animals had a worse performance, by showing a lower number of right answers, of eaten rewards and taking more time to find the first reward and to finish the task. Importantly, these differences between the two groups were only evident in the first days of testing since by the end of training all animals showed similar performances. This evidence, and the fact that most parameters significantly improved throughout the 5 testing days, allow us to conclude that all animals, both stressed and controls, adequately learned the task.

When analyzing the hole board results, two other parameters, the number of wrong answers and of repetitions after eating a reward are often used as a surrogate to

assess an animal's reference and working memory, respectively. Thus, a good score in these parameters would indicate that the animal's reference and working memory is intact, and a worse score would be an indication of stress-related damages to the PFC and hippocampus, and possibly a consequent impairment of these types of memory. Therefore, the finding that CON animals had a higher number of wrong answers and repetitions after reward, was somewhat puzzling. However, although no direct measurement of locomotor activity was performed (since the hole board was custom made and no videotracking was used), "live" observation of the tests led us to hypothesize that stressed animals, particularly in the first days, had a markedly decreased exploratory behavior that could explain, at least in part, such results. Indeed, we could observe that these animals explore the new environment (the HB apparatus) much less than the CON animals, and had a tendency to "freeze" and stay on the corners of the board when frightened. Since the STR animals explore less, it is comprehensible that they answered wrong fewer times and repeated fewer holes, since they visited fewer holes.

5.2. Electrophysiology

The occurrence and strength of LTP in the hippocampus-to-PFC pathway reflects synaptic plasticity in the PFC [57]. In 2007, Cerqueira [46] demonstrated that chronic stress impairs the development of LTP within the PFC-hippocampus circuit. Synaptic plasticity is one of the key mechanisms of learning and behavioral adaptation [57], and hippocampal derived synapses on

the PFC neurons have been shown to be involved in PFC-dependent short term memory tasks [59].

Our results confirmed that stress impairs synaptic plasticity in the PFC-hippocampus circuit. Furthermore, and more importantly, they showed that, albeit the inexistence of significant differences, stressed animals that performed the hole board task recovered some synaptic plasticity, since they presented a signal potentiation fairly higher that the STR animals.

5.3. Morphological analysis

As mentioned in the methods section, this morphological analysis of the PFC and hippocampal neurons allowed us to assess the morphological state of the neurons and deduce from there the effects of stress and of cognitive enrichment on these areas.

In the PFC's basal dendrites, the main finds were that although there are differences between the three groups concerning the number of dendrites, branches and dendritic ends, those differences were not statistically significant. On the other hand, the spine density was significantly different between CON and STR and between CON and SHB animals.

This led us to think that there is some kind of recovery of stressed animals after performing the HB task, and it raises two questions: either the training was

not long enough for the neurons to fully recover their morphology or there is a threshold on how much the neurons can recover. Still, the dendritic spine density might be more "visible" because changes in the spines occur faster that in whole dendrites.

On the apical dendrites, the difference of branch numbers between CON and STR and between STR and SHB were significant. Both proximal and distal apical regions showed a significant difference between the 3 groups in spine density. This is concordant with some studies like Radley 2004 [44], where the author states that repeated stress induces apical dendritical retraction and spine loss in layers I/II of mPFC neurons; and studies from Wellman [60] and Brown [61], which conclude that chronic stress induces profound atrophy and remodeling of the apical, but not the basal, dendrites of pyramidal neurons of layers II/III of the PFC, changes which result in reduction of average spine density of the more superficial part of the dendritic tree [60,61]. Still, Radley stated that the effects of repeated stress on structural plasticity were most prominent in distal apical dendrites [44].

Analyzing the hippocampus, the differences between the 3 groups were more prominent. There were significant differences in all parameters, except in the number of thin and thick spines. A possible interpretation for this is given by a study that demonstrated that stress-induced functional deficits propagate from a hippocampus-dependent task to a PFC-dependent task [46]. Furthermore, it is known that the hippocampus is an especially plastic region of the brain [48]

which can give us a clue about why the effects on the hippocampus are more visible than on the PFC.

Regarding the unexpected results on the number of thin and mushroom spines, the fact that there were no differences between groups and the discrepancies with other studies can be explained by two reasons, pointed out by Cerqueira in his 2006 paper [38]. First of all, spine densities vary throughout different portions of the dendritic arbor of mPFC neurons [15], so the use of randomly chosen segments in two regions of the dendritic tree might not allow for a generalization of densities in the entire dendritic tree. Secondly, the Golgi impregnation techniques assess almost exclusively spines that appear perpendicular to the dendritic shaft and parallel to the plane of the section [38]. For these reasons, an estimation of spine density and number in PFC pyramidal neurons is significantly lower than an estimation obtained with more robust techniques such as 3D reconstruction using the confocal microscope [15].

In conclusion, this worked showed that cognitive enrichment, in particular the hole board task, can revert some effect of stress, including loss of synaptic plasticity and neuronal atrophy on the hippocampus-to-PFC connection. This cognitive task was hippocampus-dependent but requested also some memory formation that is typical of the PFC. These two areas are critical on the formation of new memories and on learning processes, and this is why it is fundamental to understand how they function individually and cooperate with

each other, and also how we can revert the negative effects of stress in these areas.

The next step on this path could be to further assess the morphological correlate, i. e., volume and cell number measurements of both areas in stressed animals after cognitive enrichment. Other hypothesis would be to assess the same parameters in different time points, by prolonging the HB and comparing animals that were trained for 5 days, 7 days, 9 days, for example.

6 | References

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7 | Annexes

Annex 1

Figure I shows brain slices of control animals, illustrating right and wrong positioning of the electrodes (see methods – Electrophysiology).

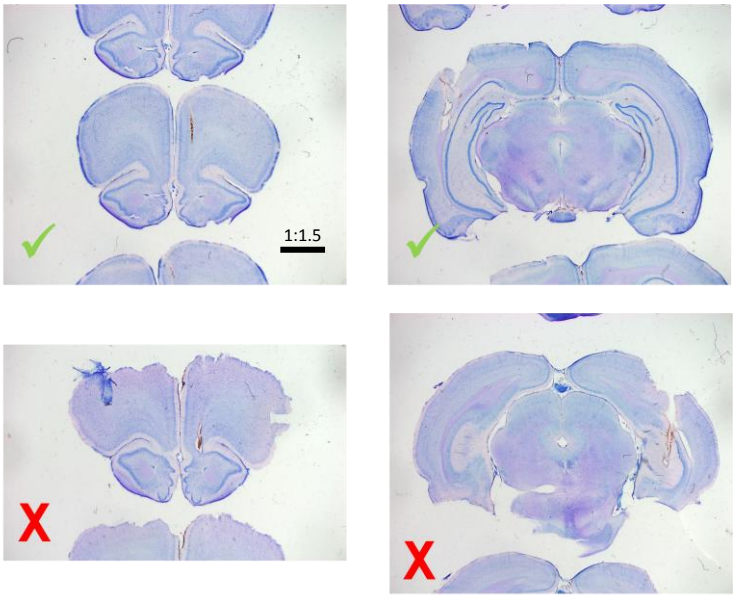

Figure I – Brain slices, illustrative of the electrode position on the brain (right position- ✓; wrong position – X). The left column shows the PFC electrode marking and the right column the hippocampus electrode marking.

Annex 2

The figure II shows a reconstructed neuron (pyramidal neuron of the PFC) on Neurolucida, and the representation of this software's tool "quick measure circle" that allowed us to choose the fragments for the spines analysis (see methods – Morphological analysis).

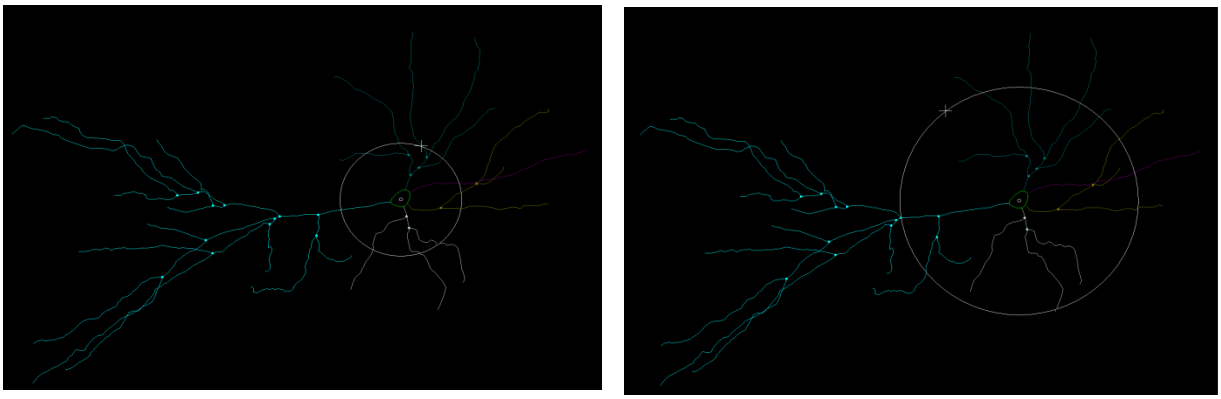

Figure II – Reconstructed neuron, as seen on Neurolucida software. The left and right circles have radii of 50 and 100 µm respectively.