Corticotropin-releasing hormone receptor 1 in the prefrontal cortex conveys acute stress-induced executive dysfunction

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Science has gone a long way toward helping man to free himself from the burden of hard labor; yet, science itself is not a liberator. It creates means, not goals. It is up to men to utilize those means to achieve reasonable goals.

Albert Einstein, 1940

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I. ABSTRACT

The prefrontal cortex (PFC) constitutes a brain region essential for the appropriate functioning of high order cognitive processes, also known as executive functions, which are required for the successful performance of our everyday activities and our adaption to dynamic environments. Furthermore, stress has been shown to affect the function and structure of the PFC. Among numerous candidates corticotropin releasing hormone (CRH), through the activation of CRH receptor 1 (CRHR1), is one of the main effector systems by which stress unchains deleterious effects upon different brain regions. However, the contributions of this system to the effects of stress in the PFC are still unclear. In the present thesis, I was able to establish the effects of acute stress in two PFC-mediated functions: Temporal order memory and reversal learning. Also, I determined the spatial distribution and differential expression of CRH- and CRHR1-containing neurons along the PFC. Additionally, I showed that acute stress induces increases in CRHR1 mRNA in the PFC, and that intra-PFC CRHR1 deletion abolishes the acute stress-induced executive dysfunction. Moreover, intra-PFC CRH microinjections mimic the effects of acute stress in temporal order memory and reversal learning in a CRHR1-dependent manner. In addition both, acute stress and intra-PFC CRH microinjections, activate the protein kinase A (PKA) signaling pathway leading to the phosphorylation of the transcription factor CREB. Finally, PKA blockade reverses the intra-PFC CRH-induced executive dysfunction. Taken together, these results suggest a molecular mechanism that links acute stress to executive dysfunction via CRHR1 activation. Thus, such findings open new intervention strategies for the development of novel therapeutic targets, which could prevent the deleterious effects of stress upon the execution of complex cognitive functions.

II. INTRODUCTION

II.1. Challenges in Modern Society

Throughout evolution, constant changes in environmental conditions triggered a set of adaptive processes which favored the development of different systems, eventually leading to increases in the complexity of already existing structures. In this context, the emergence of the central nervous system allowed organisms to execute complex reaction patterns in response to different sets of stimuli (Harris and Zahavi, 2013; Mashour and Alkire, 2013). As a result, interactions between individuals and their surrounding environment also became more intricate, placing a new set of demands that further guided biological fitness. Thus, the implementation of diverse strategies to permit individuals a successful adaptation to specific situations became a necessity (Albantakis et al., 2014).

Along this line, several physiological and structural modifications within the central nervous system improved behavioral adaptation through increases in cognitive performance. Thus, such conditions gave rise to a diverse set of skills and functions that significantly increased the behavioral repertoire and facilitated challenge coping. Consequently, animals gained the capacity to quickly process higher amounts of information in a complex fashion to accurately deliver specific responses in a context-dependent manner (Mattei, 2014; Whishaw and Karl, 2014). Among this wide cluster of abilities, many cognitive processes have been highlighted as key elements mediating behavioral efficiency. On one hand, the dynamic scenarios faced by different organisms soon placed a great demand on the faculty to change behavior according to previous experience (Jablonka et al., 2012). Such type of behavioral plasticity could only be possible by the nervous system´s capacity to be modified by experience, a capacity based on the competency to encode, store and retrieve relevant information. In other words, memory processes proved to be very useful tools to accurately deal with environmental contingencies essential for survival (Van et al., 2013).

Naturally, to successfully create reliable representations of past events within the central nervous system, it is first necessary to possess a strong ability to focus and select salient stimuli. As a matter of fact, attentional processes constitute the basis upon which memory takes place. Without a solid capacity to properly select, focus and direct attention towards biologically significant information, no memory trace can be formed (Smith et al., 2014).

Hence, the appropriate management of attentional resources by the central nervous system played also a key role in behavioral adaptation (Traub and Elepfandt, 1990).

Certainly, in order for such cognitive functions to be executed properly, an adequate structural and physiological substrate should be present. In fact, different brain regions have been shown to be responsible for such processes. Several lines of evidence have already demonstrated the essential role played by the hippocampal formation and its projections in diverse memory processes, ranging from encoding of information to its storage and retrieval (Deadwyler et al., 2014; Stollery and Christian, 2014). In addition, other reports in the literature have established the involvement of thalamic networks, brainstem monoaminergic nuclei and prosencephalic areas in the accurate allocation, distribution and execution of attentional resources (Deadwyler et al., 2014; Mohanty et al., 2008; Tsanov and OMara, 2014). Thus, intricate interactions between environmental factors, neurophysiology and neuroanatomy give rise to a diverse spectrum of functions that effectively determine adaptive processes in dynamic scenarios.

As cognitive performance improved and the brain´s structure allowed for such complex processes to take place, organisms significantly modified their interaction patterns and added further layers of complexity to our already demanding environment. As a result, cultural evolution surpassed biological evolution and a new set of problems and needs aroused, placing additional pressures upon our biological systems. In fact, modern society constitutes a highly dynamic and challenging context, characterized by the increasing application of sophisticated technologies that place a great and growing demand on the rapid and accurate processing of multimodal sensory information. Such information streams clearly exceed the brain´s performance capabilities and represent a major challenge for successful adaptation (Adey, 1997). Naturally, failure to respond properly can have relevant consequences. Basically, in order to effectively cope, one must be able to efficiently attend several stimuli simultaneously, while performing diverse tasks that also depend on the accurate anticipation of future consequences and events. As a result, an information overload takes place with a subsequent decrease in cognitive performance that eventually affects our routine activities in everyday life (Tanaka et al., 2012).

Society is by definition social, and thus social interactions constitute a key element that determines effective adaptation. However, the social relations that currently pervade our civilization are far from simple. In fact, in order to succeed and eventually achieve integration into society, optimal discrimination and anticipation of the hierarchies

dominating each social context are essential (Gibson et al., 2014). Additionally, the upcoming socioeconomic changes in living conditions, working hours and shift working have led to a series of relevant consequences including emotional dysregulation, instability, anxiety and confusion. Moreover, such conditions increase vulnerability to mood and anxiety disorders (Motomura and Mishima, 2014). In other words, modern society is characterized by a rapid pace of life, high demands, increased efficiency and competitiveness in a global economy. Together, such constrains induce a significant lack of rest, recovery and restitution which represent major risks for both mental and physical health (Lundberg, 2005). Furthermore, several alterations in socioeconomic standards have also led to relevant alterations in working conditions that strongly impact health. Thus, high quantitative job demands combined with low social support, decreased job control, low job satisfaction and increases in job strain, resulting in a lack of balance between job demands and job control eventually induce deleterious effects on health status, leading for example to hypertension (Rosenthal and Alter, 2012).

As expected, several lines of evidence have established the effects of such challenging and overwhelming socioeconomic burden in the physical and mental health of vulnerable populations, leading to the development of physiological and psychiatric disorders that strongly impact quality of life (Harb et al., 2014; Meyrer et al., 2009). However, the majority of persons seem to remain resilient to the emergence of these conditions. Nonetheless, evidence suggesting an impact on overall cognitive performance in normal population is increasing (Friedler et al., 2014). Therefore, it is highly possible that sporadic exposure to highly demanding scenarios bring a cluster of cognitive alterations with a measurable impact in everyday life.

II.2. The Prefrontal Cortex

The neuroanatomical features, functional properties, and physiological characteristics of specific brain areas support discrete cognitive processes that allowed organisms to properly cope with their surroundings. Under diverse and dynamic circumstances, a great demand is placed on neural circuits responsible for the organization of broad physiological responses and complex behaviors. Thus, a structure capable of integrating different sensory modalities and accessing the information already processed by different areas certainly increases behavioral efficacy, adding further layers of complexity to the already existing behavioral repertoire. In this context, the prefrontal cortex (PFC) is a privileged brain

region, providing multimodal integration and synthesizing actual information and past experience into a coherent framework (Kolb et al., 2012). Basically, it is one of the last cortical regions to develop both phylogenetically and ontogenetically, and reaches maximum development in the human brain, constituting almost 30% of the total cerebral mantle (Riga et al., 2014).

II.2.1. Anatomy

Many of the intricate cognitive functions that characterize the PFC are strongly supported by its unique anatomical traits and complex connectivity patterns. Hence, in order to fully understand PFC-mediated cognitive performance, it is first necessary to elucidate its neuroanatomical properties. In humans, the frontal lobe lies anterior to the central sulcus and can be divided into two major parts, a caudal part containing the motor cortex and a rostral part comprising the PFC. The PFC is located at the anterior end of the cerebral hemispheres and extends till the frontal pole (Jeon, 2014). On the medial surface, the PFC lies anterior to the medial component of the motor cortex as well as the rostral portion of the cingulate gyrus. The primate PFC is usually divided on broad anatomical regions. Its major subdivisions encompass the ventrolateral, dorsolateral, medial and orbitofrontal cortices. A few landmarks help to accurately segregate the aforementioned areas. For instance, the principal sulcus, located on the lateral surface of the hemispheres, separates the ventrolateral from the dorsolateral PFC (Spencer et al., 2014). In addition, as its name suggests, the medial PFC is located on the medial surface of the hemispheres. Finally, the orbitofrontal PFC lies in the anterior cranial fossa above the bony orbit, establishing the basal, or ventral, surface of the frontal lobes (Dobrossy et al., 2014) (Figure 1). Concerning its cytoarchitecture, a large portion of the PFC has been classified as granular cortex and contains six well defined layers, including a prominent external granular layer II and internal granular layer IV (Tremblay et al., 2014).

II.2.2. Connectivity

Besides the structural and cytoarchitectural properties of the PFC, it is also relevant to highlight its connectivity patterns with a constellation of cortical association areas. In fact, the PFC is well known for establishing widespread cortico-cortical networks with distal parts of the cerebral cortex, particularly uni and multi-modal association areas (Figure 2). Whereas unimodal association cortex integrates sensory information pertaining to one sensory modality, multimodal association cortex synthesizes highly processed information whose source can be traced back to multiple sensory modalities (Helie et al., 2014). The

PFC is linked to association cortices of the parietal, temporal and occipital lobes. In this regard, several anatomical and behavioral studies have shown long association pathways reciprocally linking the posterior parietal cortex with the PFC, responsible for the appropriate execution of visually guided movements. Therefore, the dorsolateral PFC has been suggested to process information concerning where an object is in space (Tse et al., 2014). It is also known that both, the inferior parietal and ventrolateral temporal project to the ventrolateral PFC. Such circuits provide data regarding form and object recognition. Hence, the ventral pathway seems to constitute a processing stream that addresses *what* an object represents in the extrapersonal environment (Ketz et al., 2015). In addition to these long association circuits, there are also short association networks that interlock the different subdivisions of the PFC with one another in an organized fashion (Yang et al., 2014). These broad set of interconnections between the PFC and different regions along the neuraxis give rise to a cluster of higher order cognitive functions. They include working memory, motor planning, development and implementation of long-term strategies, decision making and problem solving among others, thus playing an essential role in behavioral organization and modulation (Bourguignon, 2014; Pistillo et al., 2015).

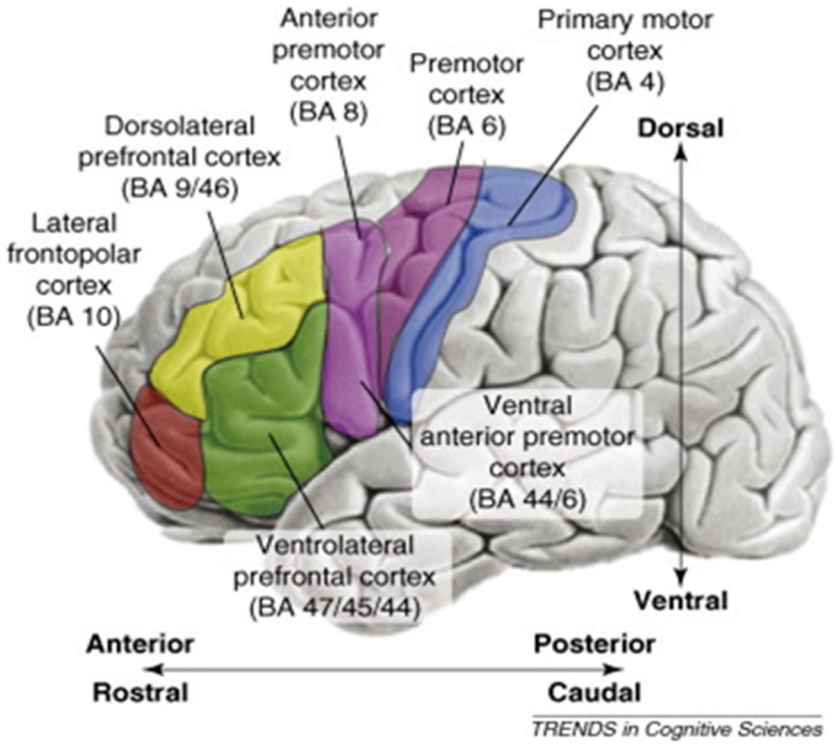

Figure 1. PFC anatomy.
Schematic diagram depicting the major anatomical sub-divisions in the frontal lobes, encompassing the PFC. Boundaries and Brodmann areas (BA) are only approximate. Arrows indicate anatomical directions of anterior/rostral (front) versus posterior/caudal (back) and dorsal (up) versus ventral (down) (Badre, 2008; Pistillo et al., 2015).

In addition to the neuroanatomical connections that support the cognitive functions subserved by the PFC, it has also been established that the PFC possesses strong structural interactions with the limbic lobe, arising from the cingulate, orbitofrontal, temporopolar, perihinal, entorhinal, posterior parahippocampal and insular cortices (Wolff et al., 2014). Most of these pathways project mainly to the posterior orbitofrontal and medial prefrontal regions and many of them have been shown to be reciprocal. As well, robust projections from the basolateral and accessory basal nuclei of the amygdala to the posterior orbitofrontal and medial PFC have been described (Herry and Johansen, 2014). The amygdala constitutes a convergent recipient of several cortical inputs from different association areas, including both the auditory and visual cortex. Consequently, it is likely that amygdalar outputs towards the PFC help to shape information related to the interplay between the internal and external environments of the organism (Cassaday et al., 2014).

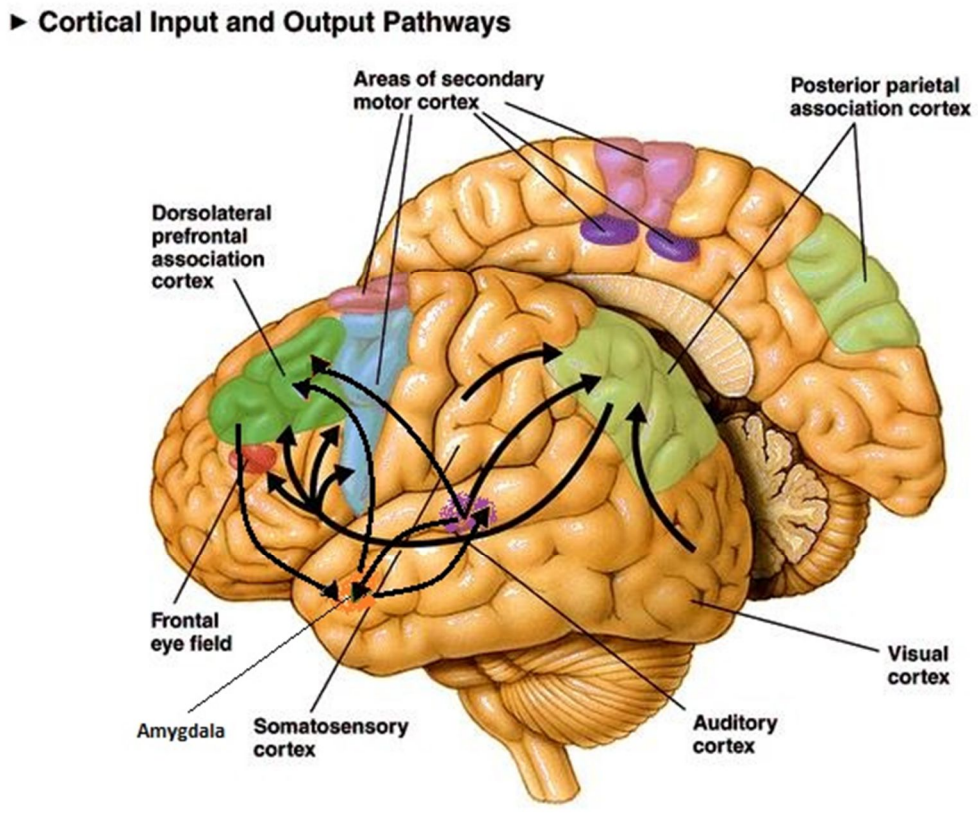

Figure 2. PFC Connectivity
Schematic diagram depicting the different pathways that connect the PFC with several cortical regions. Modified from Paskari, 2007.

II.2.3. Function

Considering the conglomerate set of afferents received by the PFC together with the abundant number of bidirectional interactions shared with several brain areas, the PFC holds a strong potential to mediate highly complex cognitive functions. As a matter of fact, various lines of evidence have shown that the PFC is responsible for accurately planning future behavior and anticipating possible consequences, thus executing responses in a goal directed fashion. In other words, the PFC is involved in the temporal organization of behavior, supporting cognitive functions necessary to arrange behavior in time and context, allowing the organism to generate behavioral strategies to enhance environmental adaptation (Puig et al., 2014). Such role extends to all domains of voluntary action, including skeletal movement, ocular motility, speech and even logical reasoning (Sebastian et al., 2014). Basically, a great demand is placed on PFC networks once the individual is required to assemble temporally extended sequences of deliberate action, particularly when the action to be performed is new and surrounded by high levels of uncertainty. This is achieved through a temporally retrospective function of short-term memory combined with a prospective function of preparatory set for action. These two processes allow the PFC to continuously reconcile past and future events during the course of behavioral execution, keeping action in logical order and on target (Goldman-Rakic, 1994) (Figure 3).

In addition, several studies have also characterized the role played by the PFC, particularly the orbitomedial region, in the inhibitory control of interference. In other words, the PFC is also responsible for the correct suppression of extraneous (sensory) or internal distractors. This kind of control normally improves and facilitates the attentional processes of the organism to relevant inputs and to the pursuit of goals (Weinberger et al., 1994). When this function fails, the subject becomes excessively distractible, incapable of suppressing the interference from trivial, irrelevant, or inappropriate stimuli from the internal or external milieu. Then, behavior is easily diverted from its original goal and tasks are not carried out to completion. Furthermore, such distractibility is usually accompanied by motor disinhibition, giving rise to a syndrome that resembles the one exhibited by children with Attention Deficit Hyperactivity Disorder (Tsutsui-Kimura et al., 2014).

Based on this, it is only natural that lesions or disruptions of the PFC have a strong impact on quality of life and the ability to properly cope with environmental challenges. For instance, it is known that PFC damage can lead to deficits in sustained and selective attentional processes. Thus, aside from general disinterest, patients with PFC injury show

difficulties in directing attention to particularly relevant information in the external setting (Arnsten, 2009). This is expressed through shortened attentional spans with problems to concentrate and maintain attention on any given item of information. Consequently, this deficit in the temporal continuity of the focus of attention gives rise to troubles in short-term memory and preparatory set, weakening the capacity to retain new information for prospective use in the absence of environmental stimulation, also called working memory (Funahashi et al., 1989).

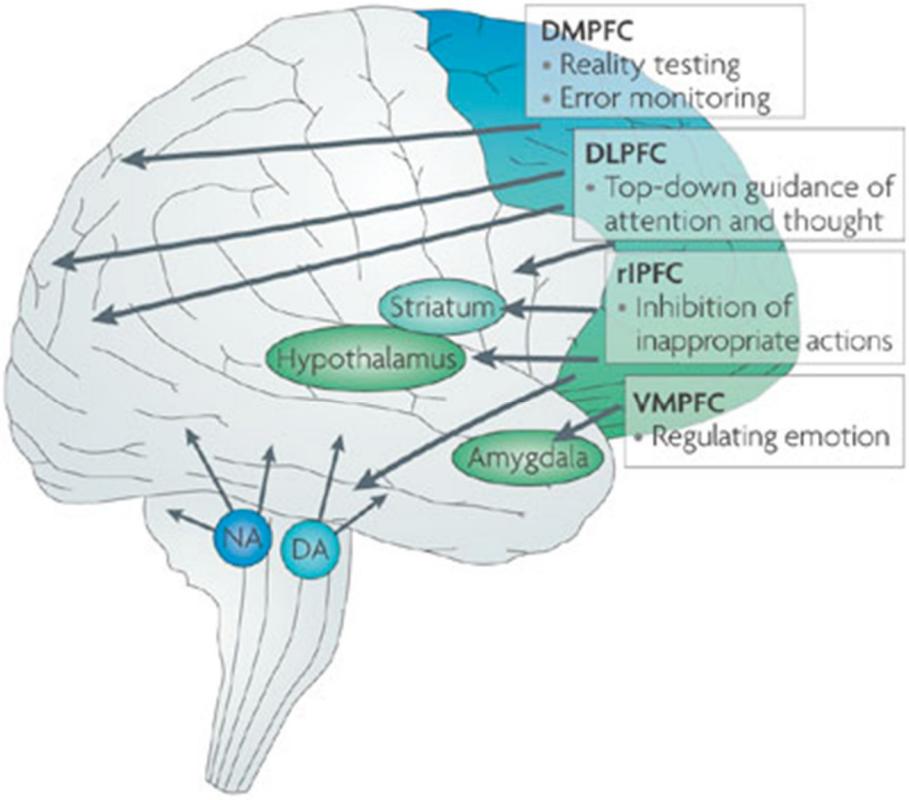

Figure 3 PFC function.
Schematic diagram depicting the different cognitive functions exerted by the PFC together with the corresponding neuroanatomical connections supporting such processes. The dorsolateral PFC (DLPFC) has extensive connections with sensory and motor cortices and is key for regulating attention, thought and action. The right inferior PFC (rIPFC) seems to be specialized for inhibiting inappropriate motor responses. By contrast, the ventromedial PFC (VMPFC) has extensive connections with subcortical structures (such as the amygdala, the nucleus accumbens and the hypothalamus) that generate emotional responses and habits and is thus able to regulate emotional responses. Finally, the dorsomedial PFC (DMPFC) has been associated with error monitoring and reality testing. These PFC regions extensively interconnect to regulate higher-order decision making and to plan and organize for the future (Arnsten, 2009).

II.2.4. PFC Dysfunction

Perhaps one of the key hallmarks that characterize PFC dysfunction is a difficulty to properly plan behavior. Therefore, individuals are no longer capable of planning any novel structures of action in an organized fashion, keep them in mind and execute them. Despite the fact that such impairment permeates all action domains, it is more evident at the highest level of speech, behavior and rational thinking (Vadovicova, 2014). Hence, patients with PFC damage cannot plan a new discourse and perform it, anticipate substantial movements or departures from daily routines and elaborate novel trends of thought leading them to logical conclusion. Basically, the planning deficit exhibited by these patients constitutes an alteration in the representation and preparation for future action (Verschure et al., 2014). Additionally, PFC disruption also causes speech disorders attributable to a malfunction of temporal integration, characterized also by an impoverishment of verbal expression, spontaneous speech and verbal fluency. Basically, speech disorders arising from PFC lesions can be ascribed to impairments in the capability of synthesizing verbal expression in the temporal dimension, which depends on the interplay between memory and the preparatory set for action. Furthermore, PFC dysfunction can also have a strong impact on behavior, leading to a temporal concreteness in which behavior remains anchored to the "here and now". In other words, behavior seems deprived of perspective either back or forward in time, driven by routine and devoid of creativity (Ries et al., 2014).

Besides impairments in interference control, PFC damage can as well lead to a general disinhibition of motor activity, thought and impulses. The resulting hypermotility gives the appearance of an excessive drive, accompanied by difficulties to stop ongoing behavior or concentrate on particular items. These subjects seem to be driven by an incessant need to act in a hazardous and purposeless fashion. Furthermore, this behavioral profile interferes with sleep, logical thinking and basic drives (Donnelly et al., 2014). Thus, patients tend to exhibit hyperfagia and hypersexuality, disregarding common good judgement. Not surprisingly, these symptoms together with the euphoria that usually accompanies this syndrome can be easily mistaken for mania (Sebastian et al., 2014). Summarizing, the cognitive control and supervisory action carried out by the PFC have given rise to the term "executive system", which performs a continuous management of cognition and behavior. In addition, the different cognitive and behavioral operations implemented by this system are now regarded as "executive functions" and play a key role in the dynamic adaptation demanded by modern society (Bagshaw et al., 2014).

II.2.5. PFC and psychiatric disorders

Given the relevance of executive functions to successfully cope with the diverse challenges faced in our everyday life, several studies have pointed towards a robust involvement of the PFC networks in the etiology of different psychiatric disorders that strongly impact quality of life. In this regard, one of the core symptoms that characterize schizophrenia is a failure in the proper temporal integration of thought. Such deficits are commonly evidenced by the constant blocking and looseness of associations. Thus, functional neuroimaging approaches have shown abnormally low levels of frontal metabolism in schizophrenic patients, particularly when faced with neuropsychological tasks that demand PFC activation (Opris and Casanova, 2014). On the other hand, there are certain conditions in which a hyperfunction of the orbitomedial PFC can give rise to an excessive inhibitory control of cognition and behavior. In these cases, attention is abnormally riveted to specific contents of thought or behavioral patterns, which become overpowering and end up implacably controlling the patient´s mind and behavior. This is what is usually referred to as obsessive compulsive disorder (OCD). In fact, imaging studies have shown orbitomedial PFC hypermetabolism in OCD patients (Nakamae et al., 2014). Additionally, it has been shown that cerebral gray matter reductions in specific regions, particularly the ventromedial PFC, can act as a crucial pathobiological marker for Post-Traumatic Stress Disorder (Kuhn and Gallinat, 2013). Furthermore, several lines of evidence point to a strong role of the PFC in affective disorders such as depression. Hence, decreased functional activity in specific subregions of the PFC seem to give rise to cognitive alterations such as ruminative thoughts, biased attention, biased processing and biased memory for negative stimuli, which eventually lead to the emergence of depressive symptoms (Disner et al., 2011).

II.2.6. Animal studies

Besides human studies, several lines of evidence have also pointed out the anatomical, physiological, neurochemical and functional properties of the PFC in other species. As a result, a cross-species functional and anatomical homology for this brain region has been suggested (Holmes and Wellman, 2009). Initial definitions of the PFC were very restrictive, using the target of the cortical projections from the dorsomedial thalamic nucleus as single defining criteria to identify the PFC. Accordingly, the general statement concerning the PFC was that it was exclusively present in primate species. However, as previously shown, single criteria do not seem to be sufficient to define this complex structure. Therefore, additional anatomical and functional properties must be taken into consideration to

properly recognize the PFC in non-primate species. Certainly, the rodent PFC is not as differentiated as it is in primates. However, anatomical dorsolateral-like features have been established in frontal cortical area 2 and anterior cingulate (AC) regions of the rodent brain, particularly concerning PFC–basal ganglia–thalamocortical circuits together with the reception of amygdalar inputs. Furthermore, similarly to macaque PFC, the rodent PFC also receives multimodal cortico-cortical projections from motor, somatosensory, visual, auditory, gustatory and limbic cortices (Van Eden et al., 1992). Also, both species exhibit a robust cholinergic innervation arising from the basal forebrain nuclei together with reciprocal connections between the medial PFC (mPFC), locus coeruleus, medial and dorsal raphe nuclei. Summarizing, in the rodent, functions of the dorsolateral and ventromedial PFC are thought to be integrated in the phylogenetically ancient mPFC (Uylings et al., 2003; Vertes, 2002). Thus, the mPFC in the rodent encompasses a large area of the forebrain, anterior to the genu of the corpus callosum and extending rostrally until the olfactory bulbs. It is comprised of the AC, prelimbic (PL) and infralimbic (IL) regions (Holmes and Wellman, 2009).

Concerning the functional aspects of the rodent PFC, it is important to consider that each species has a unique behavioral repertoire that allows it to survive in a specific environmental niche. Hence, it is highly likely that neocortical organization uniquely reflects patterns of behavioral adaptation pertaining to a particular species. Thus, care should be taken in making direct comparisons with the primate PFC. Nonetheless, certain traits and capacities remain common and are actually shared by all mammals. For instance, they must be able to detect and interpret sensory information, relate it to past experience and act appropriately. Therefore, they must be able to use sensory information to properly guide behavior on line, for example in the visuosomotor control of movements in space or the identification of food items using visual, tactile and olfactory information. Consequently, in rodents, there also seems to be a type of master control system responsible for selecting behavior and generating behavior according to past experience (Kolb and Whishaw, 1983). Accordingly, executive functions measurable in rodents include working memory, cognitive flexibility, sustained attention, temporal order memory and inhibitory response control (Holmes and Wellman, 2009).

Moreover, lesional approaches have evidenced similarities between PFC-related performance in rodents and primates. For instance, damage to the mPFC produces severe deficits in acquisition and retention of working memory tasks (Broersen et al., 1995;

DeCoteau et al., 1997; Kolb et al., 1974; Wikmark et al., 1973) . Additionally, impairments have also been demonstrated in various types of attentional tasks (Muir et al., 1996). Likewise, disruptions in properly executing and ordering motor sequences such as nest building, food hoarding or latch opening have been established after mPFC injury in rodents (Kolb et al., 1974; Kolb and Whishaw, 1983). Also, deficits in the generation of new behavioral rules together with an inability to inhibit previous predominant responses have been observed after unilateral dorsomedial PFC lesions in mice. Such perseverative pattern exhibits striking similarities with the effects of PFC lesions shown in monkeys and humans, suggesting once more the relevance of PFC networks in different mammalian species (de Bruin et al., 1994).

Perhaps one of the most studied paradigms in rodents concerning PFC disruption refers to fear extinction. On this particular experimental approach, both ventromedial and dorsomedial PFC regions seem to play different roles concerning the emotional regulation of behavior. Thus, whereas dorsomedial lesions induce deficits in the expression of a conditioned fear response, ventromedial damage impairs the proper inhibition of a previously learned fear response after extinction training. These findings strongly posit the emotional regulation exerted by the rodent PFC, indicating once more its resemblance with the functional properties of the human PFC. Furthermore, the role exerted by the rodent PFC on the temporal organization of behavior has been elegantly demonstrated employing a disconnection analysis between the mPFC and the perirhinal cortex, a region involved in memory coding and consolidation. Hence, using unilateral and bilateral lesions of either one or both structures, it was established that the mPFC is essential for the accurate discrimination of the temporal order in which past events take place, also called temporal order memory (Barker et al., 2007). Naturally, such process is fundamental for the correct organization of behavior in time and space. Once more, such results evidence the parallel existing between the rodent and human PFC, underlining its relevance for the proper execution of structured responses, leading to increases in the efficacy of adaptive processes. In general, it seems that whereas the rodent PL region is involved in attentional, working memory and response selection functions, the AC is involved in generating rules associated with behavioral organization and motor sequencing. Taken together, such data indicates a strong convergence of class-common features across mammals.

II.3. The prefrontal cortex under stress

Given the fundamental involvement of the PFC in the dynamic interactions between the individual and its environment, it is only natural that this region also responds to external events by undergoing physiological and structural modifications. Indeed, it has been established that particular circumstances can actually shape many of its anatomical features and induce significant alterations in its basic functions. Thus, despite the fact that the PFC networks can accurately guide behavior according to past experience, it has also been shown that this prosencephalic structure can be very sensitive to adverse events that threaten the ability of the organism to adapt and cope, also called stressors (Kolb et al., 2012). Basically, such situations evoke a set of physiological reactions that through the activation of specific endocrine responses affect the whole individual and alter the function of several systems within the body, including the PFC (Popoli et al., 2012a). In other words, several lines of evidence have indicated that the neuromodulatory changes that characterize the stress response can rapidly disrupt PFC network connections and markedly impair PFC processes.

II.3.1. Structure

On a structural level, daily sessions of 3 hours of restraint stress over a period of 3 weeks induce dendritic remodeling in the apical branches, with dramatic reductions in branch number and length in terminal branches distal to the soma within the PFC of rodents (Cook and Wellman, 2004). In addition, this stress-induced debranching of apical dendrites is associated with a significant decrease in the density of dendritic spines (Michelsen et al., 2007; Radley et al., 2008). Further studies have actually determined that such stress-induced anatomical alterations can also be achieved by administering milder stress protocols, characterized by a single stress episode, suggesting that the PFC possess a higher sensitivity to stress, compared to other regions (Brown et al., 2005; Izquierdo et al., 2006). Moreover, one 30-min bout of elevated platform stress is able to impair a long form of long-term potentiation in the PL (Maroun and Richter-Levin, 2003). Taken together, these findings indicate an exquisite morphological and physiological susceptibility of mPFC neurons to stress.

II.3.2. Neurotransmission

On the other hand, there is also clear evidence showing robust effects of stress on the activity of different neurochemical candidates that eventually modify PFC physiology.

Among many neurotransmitter and neuromodulatory systems affected by stress within the brain, increases in glutamatergic neurotransmission seem to have a particularly strong impact on PFC function. Glutamate constitutes the main excitatory neurotransmitter in the brain and is synthesized *de novo* from glucose in astrocytes (Popoli et al., 2012b). Once synthetized, it is stored in presynaptic vesicles within neurons and its exocytotic vesicular release is tightly regulated through the so-called SNARE complex, formed by the interaction of two synaptic membrane proteins and a vesicular protein. After neuronal excitation, this cluster of proteins mediates the fusion of the synaptic vesicles with the presynaptic membrane, leading to glutamate release (Lang and Jahn, 2008). After release, glutamate regulates post-synaptic activity through the stimulation of metabotropic receptors and the ionotropic glutamate receptors AMPA and NMDA, among others (Sudhof and Rothman, 2009).

Several lines of evidence have determined significant increases in glutamate release in the PFC after different types of acute stressors, including tail pinch, forced swim or restraint stress (Reznikov et al., 2007; Venero and Borrell, 1999). Similarly, other studies have also demonstrated stress-induced effects on depolarization-evoked glutamate release in the PFC, suggesting relevant changes on the excitatory activity of the PFC networks after stress (Musazzi et al., 2010). At the level of the presynaptic machinery, increases in the number of SNARE complexes bound to the presynaptic membrane have been observed after stress induced by a footshock. Together with such effects on presynaptic glutamate release, additional reports have also shown a delayed and sustained impact of acute stress on postsynaptic glutamate receptor-mediated responses in the PFC (Yuen and Yan, 2009). Thus, electrophysiological approaches have shown increases in NMDA and AMPA-mediated synaptic currents in PFC pyramidal neurons after acute stress, caused by an enhanced surface expression of receptors and their different subunits at the postsynaptic plasma membrane. This suggests that acute stress can lead to an increased delivery of glutamate receptors from intracellular or extrasynaptic surface pools to the synaptic membrane in the PFC. Additionally, acute stress also seems to impair synaptic plasticity in PFC circuits, modifying the efficacy of glutamate transmission. For instance, it is known that acute stress can inhibit long-term potentiation (a measure of increased efficiency in glutamatergic transmission) in the amygdala-PFC and hippocampus-PFC pathways (Rocher et al., 2004; Yuen and Yan, 2009).

Besides the glutamatergic system, several studies have also established relevant effects of acute stress in other neurotransmitter systems within the PFC. For instance, it is known that acute stress exposure can induce important increases in dopamine and noradrenaline release in the PFC (Finlay et al., 1995; Roth et al., 1988). On one hand, the noradrenergic modulation of PFC activity seems to be under a very delicate balance, where too much or too few neurotransmitter can have drastic effects on the functionality of the PFC networks. Furthermore, such regulation also depends on the specific stimulation of different kinds of receptors. Thus, whereas α_{2A} receptors optimize PFC-mediated cognition, α_1 and β_1 receptors have an opposite effect (Arnsten and Goldman-Rakic, 1985; Ramos et al., 2005). Thus, acute stress-induced increases in noradrenaline release would lead to the activation of the lower affinity α_1 and β_1 receptors, thus giving rise to PFC dysfunction. Consequently, stress-evoked cognitive alterations can be prevented by the intra-PFC microinfusion of α_1 antagonists (Birnbaum et al., 1999). Similarly, studies performed in humans have found that β receptor antagonists can abolish the stress-evoked impairment in cognitive flexibility (Alexander et al., 2007).

Concerning dopamine, it has been established that very mild stressors are able to evoke significant increments in dopaminergic neurotransmission in the PFC, suggesting a very low threshold for the stress-induced, dopamine-mediated effects on PFC functions (Deutch and Roth, 1990). In addition, this stress-evoked enhancement in dopamine efflux within PFC networks seems to depend on the local activation of glucocorticoid receptors in the PFC. Moreover, this effect on dopaminergic neurotransmission caused by the glucocorticoids released during a stress response has also a strong functional impact, leading to impairments in working memory (Butts et al., 2011a). Dopamine exerts its modulatory action over PFC activity mainly through the stimulation of D1 receptors (Arnsten and Goldman-Rakic, 1990; Sawaguchi and Goldman-Rakic, 1991). Particularly, the stress-evoked PFC dysfunction has been rescued using pharmacological approaches based on the administration of D1 antagonists (Murphy et al., 1996). In addition, the D2 receptor also seems to be partly responsible for the effects of acute stress on the PFC. For example, it is known that excessive D2 receptor stimulation impairs PFC-mediated cognition in humans and animals (Druzin et al., 2000; Gibbs and D'Esposito, 2005).

II.3.3. Intracellular effects

Once the activity of the aforementioned neurotransmitter systems is increased after stress, higher amounts of these neurochemical candidates become available and are released into

the synaptic cleft. As a consequence, the corresponding receptors are further stimulated and their effects are also enhanced. Besides modifying the electrophysiological activity of the cell, neurotransmitter receptors also activate a series of intracellular cascades that eventually have an effect on cell function through the modulation of genetic transcription and other cellular processes. Therefore, it is expected that the stress-induced alterations on neurotransmission ultimately lead to important changes on intracellular signaling within the PFC. In fact, different signaling pathways have been regarded as key candidates mediating the aforementioned effects of acute stress in the PFC. In this regard, it has been observed that a single episode of restraint stress causes an increase in the expression of *Arc* (activity-regulated cytoskeletal-associated protein), an immediate early gene that has a key role in activity-dependent synaptic modification (Messaoudi et al., 2007). Also, it has been suggested that the stress-induced, α_1-mediated, effects on PFC-dependent cognition are exerted through the phosphatidylinositol-protein kinase C (PKC) pathway. Consequently, intra-PFC infusion of PKC inhibitors reverses the effects of stress and α_1 agonists in working memory (Runyan et al., 2005). Conversely, stress-evoked deficits in working memory can be mimicked by the intra-PFC microinjection of PKC activators. At the cellular level, the suppression of PFC neuronal firing induced by the stimulation of α_1 receptors can be reversed by the iontophoresis of a PKC inhibitor (Birnbaum et al., 2004).

Additionally, signaling through cyclic adenosine monophosphate (cAMP) also seems to regulate the stress-induced, catecholamine-mediated, PFC disruption. As a matter of fact, the cAMP intracellular signaling cascade has been shown to exert a highly powerful impact on working memory network activity and cognitive function. Once cAMP is increased within the cell, it can activate several signaling pathways leading to a diversity of effects. One of the key targets stimulated via cAMP is the hyperpolarization-activated cyclic nucleotide-gated cation channel (HCN), and these are located on both the dendritic shafts and dendritic spines of PFC pyramidal cells. In line with this, intra-PFC HCN1 knockdown improves working memory performance (Wang et al., 2007). Accordingly, uncontrollable stress leads to excessive production of cAMP together with a concurrent impairment in PFC-dependent cognition. Such deficits can actually be rescued by blocking either cAMP or HCN channels in the PFC, or mimicked by the administration of cAMP analogues. (Taylor et al., 1999; Vijayraghavan et al., 2007).

II.3.4. Functional consequences

Concerning the functional effects of acute stress-induced PFC disruption, early observations began after the Second World War, where highly skilled pilots during peacetime often crashed their planes during the stress of battle, owing to mental errors (Arnsten, 2009). In fact, initial studies were able to show that stress exposure impaired performance of tasks that required complex and flexible thinking (Hartley and Adams, 1974). Actually, loss of PFC-mediated self-control during stress exposure can help to explain the relapse of a number of maladaptive behaviors such as drug addiction, smoking, drinking alcohol and overeating (Li and Sinha, 2008). In order to fully elucidate the effects of acute stress on PFC-dependent cognition, several animal models have been employed. In this regard, it has been established that an uncontrollable stressor induces deficits in selective attention that lead to impaired performance on a Y-maze task (Minor et al., 1984). Another study demonstrated that mild acute stress impairs the accuracy to respond, and produces a perseverative pattern, in a working memory task, mimicking the effects of PFC lesions and suggesting a stress-induced PFC dysfunction (Di Pietro et al., 2004). Conversely, performance on control tasks with similar motor and motivational demands, that do not depend on the PFC, were not altered by this stressor. Similarly, rats exposed to acute stressors also exhibited alterations in a spatial delayed alternation task, which requires the PFC. Besides these PFC-mediated cognitive processes, other executive functions are also affected by stress. Thus, different stress protocols have given rise to deficits in spatial reversal learning that also correlate with significant decreases in mPFC volume (Enthoven et al., 2008; Hill et al., 2005). Similar findings have also been obtained using an attentional set-shifting paradigm, where 2 weeks of unpredictable stress impaired reversal learning and extra-dimensional set-shifting (Bondi et al., 2008). Along these lines, stress has also been shown to alter the ability to flexibly alter behavior through the inhibition of a predominant learned response, also called fear extinction. Such effects were observed together with a significant retraction of apical dendrites in the IL, suggesting the involvement of PFC networks. Basically, extinction deficits are regarded as a form of "emotional perseveration", somehow akin to the cognitive perseveration caused by PFC lesions (Izquierdo et al., 2006). In a similar fashion, a single bout of elevated platform stress leads to fear extinction deficits accompanied by shifts in plasticity in the IL-amygdala pathway (Akirav and Maroun, 2007; Maroun, 2006).

Summarizing, stress in its diverse presentations seems to have a clearly strong impact on the morphological, physiological and functional properties of the PFC. As previously stated, such alterations seem to take place via classical neurotransmitter systems. However, it has become evident that glucocortidoids released during stress constitute direct mediators of these alterations. Nonetheless, the role played by other stress-related systems in PFC dysfunction still remains unexplored. In fact, as already mentioned, this prosencenphalic region appears to be particularly sensitive to the effects of stress, being affected by mild and even acute exposures to stressful situations. This acquires special relevance due to the fact that nowadays, many individuals routinely experience high levels of social, environmental and work-related stressors on an everyday basis (Duman and Aghajanian, 2012). Thus, it is highly probable that such conditions can lead to important reductions in dendrite complexity and synaptic density within the PFC, giving rise to an executive dysfunction that strongly impacts quality of life. However, in order to fully understand the mechanisms through which stress can alter PFC-dependent cognition, it is first necessary to elucidate the different components and steps that characterize the stress response.

II.4. The stress response and the Hypothalamus Pituitary Adrenal Axis

The stress response emerged in order to provide the required metabolic realignment essential for survival when facing challenges, thus increasing biological fitness and promoting adaptive processes. As a result, animals became suited to deal with sudden external changes, being able to modify their entire physiology to respond more efficiently to those challenges (Duman and Aghajanian, 2012; Panagiotakopoulos and Neigh, 2014). In general terms, stress can be defined as any threat, real or perceived, to homeostasis or survival. Naturally, the fast coordination and execution of extensive physiological adjustments in response to environmental threats must start at the central nervous system, where all the sensory information is conveyed and integrated. In fact, the so called stress response does not refer to a single reaction, but to a cluster of physiological alterations that affect different body systems and occur in a harmonized and systematic pattern (de Kloet et al., 2005). Hence, upon sensory integration, information must be directed at specific regulatory centers in the brain that allow the rapid initiation of a physiological cascade in an organized fashion (Panagiotakopoulos and Neigh, 2014). Actually, this set of reactions is

not carried out by a single system but by two different neurobiological circuits that together orchestrate this wide set of responses: the Hypothalamus-Pituitary-Adrenal (HPA) and the sympathetic-adrenal-medullary (SAM) axes (Myers et al., 2014). Essentially, this physiological response to stressors takes place in two temporally distinct waves. Initially, an immediate 'wave' is triggered within seconds by the SAM, giving rise to a fast increase in the levels of circulating catecholamines, thus preparing the organism for a physical emergency. Posteriorly, a slower endocrine 'wave' is coordinated by the HPA axis, resulting in the release of glucocorticoids (GC) (mainly cortisol in humans and corticosterone in rodents), allowing the long-lasting adaptations to stressors (Braquehais et al., 2012).

Given the key role played by the HPA axis in the coordination of the stress response, it is highly relevant to fully understand its anatomy. This system consists of a complex consecutive arrangement of structures, which are regulated in a reciprocal way by forward and feedback mechanisms. The hypothalamus is part of the diencephalon. It is limited anteriorly by the optic chiasm and the lamina terminalis. Posteriorly, it is demarcated by an imaginary plane defined by the posterior mammillary bodies ventrally and posterior commissure dorsally (de Kloet et al., 2005). Caudally, the hypothalamus merges with the midbrain periaqueductal gray and tegmental reticular formation. The dorsal limit of the hypothalamus is determined by the horizontal level of the hypothalamic sulcus on the medial wall of the third ventricle. Laterally, it is limited by the internal capsule and the basis of the cerebral peduncles (Smith and Vale, 2006). The activation of the stress response takes place either as a consequence or in anticipation of a challenge. On one hand, anticipatory reactions demand the organism to reference previous experiences and predict the need for energy mobilization. Basically, the hypothalamus acts as an integrator, receiving converging inputs from virtually every sensory and autonomic system related to the internal and external environment of the organism. Such process is mediated by multi-synaptic forebrain projections to the neuroendocrine cells in the medial parvocellular paraventricular nucleus (PVN) of the hypothalamus, which respond rapidly to this convergent information by modifying the activity of its projections to the median eminence (Holsboer and Ising, 2010).

The median eminence, or infundibulum, gives rise to the pituitary stalk at the base of the hypothalamus, in the floor of the third ventricle. This structure constitutes the main site where the hypothalamic neuroendocrine cells release their secretions to the primary plexus of the hypophysial portal system in order to regulate the adenohypohysis or anterior

pituitary (Hillhouse and Grammatopoulos, 2006). The anterior pituitary, like the hypothalamus, is an endocrine gland, releasing its hormones into the circulatory system, where they will act remotely at receptors in target organs of the body. The pituitary gland lies close to the medial basal hypothalamus, to which it is connected by the pituitary stalk (Del and Besedovsky, 2000). In most vertebrates it is divided into three lobes: the anterior or adenohypohysis, the posterior or neurohypophysis and the intermediate lobe. The anterior pituitary contains neurosecretory cells responsible for the synthesis of adenocorticotropic hormone (ACTH). These corticotropes constitute approximately 20% of the anterior pituitary cells and play a fundamental role in the initiation of the stress response (Bale and Vale, 2004).

It is known that the hypothalamic neurons produce neuropeptides that regulate the function of the adenohypophysis, triggering the stress response in threatening circumstances. The main factor secreted under stress stimulates the release of anterior pituitary hormones and is therefore referred to as a releasing factor. Particularly, this hormone is known as the corticotropin releasing hormone (CRH) or corticotropin releasing factor (CRF) (Korosi and Baram, 2008). This hypophysiotropic hormone is synthesized in neuronal cell bodies of the PVN, transported in dense core vesicles to nerve endings in the stalk median eminence, stored in axonal endings, released into the interstitial space upon stimulation in contiguity with the primary portal capillary plexus, and distributed to the anterior pituitary by means of the portal circulation (Steckler and Holsboer, 1999).

Once CRH is released and reaches the anterior pituitary, it binds to CRHR1 receptors. Furthermore, CRHR1 activation eventually stimulates the synthesis of the large precursor molecule proopiomelanocortin (POMC), which is then cleaved into ACTH and secreted into the systemic circulation. Thus, the hypothalamic CRH neurosecretory neurons constitute the main regulators of HPA activation, coordinating the stress response, facilitating homeostatic maintenance and promoting behavioral adaptation to environmental challenges (Bonfiglio et al., 2011). Finally, ACTH reaches the adrenal cortex and stimulates the release of glucocorticoids (GC) (Herman et al., 2012).

After GC are synthetized and released, they cause a global redistribution of energy, increasing fuel availability in order to enhance survival in the face of real or perceived threats. When secreted, GC bind to high affinity mineralocorticoid receptors (MR) or lower affinity glucocorticoid receptors (GR), which act as ligand-gated transcription factors to

positively or negatively regulate gene expression (Roozendaal et al., 2009). Hence, once bound to their ligand, GC receptors translocate into the nucleus where they bind to specific sites on the promoter region of the responsive gene. Basically, GC are key elements in the regulation of glycogen utilization and storage, playing an essential role during the alarm reaction that characterizes the stress response by promoting a highly catabolic profile. Given the energetic demands placed upon an individual under stress, GC are able to induce lipolysis, glycogenolysis and proteolysis, leading to important increases in the levels of free fatty acids, glucose and aminoacids as readily available fuels for the body. In other words, the goal is to prepare the organism for intense physical activity through the mobilization of energy resources and the improvement of the cardiovascular tone (Barsegyan et al., 2010). This is achieved through a series of physiological processes. On one hand, glucose metabolism is altered by breaking down protein into glucose and making the nutrients stored in muscles to become available, thus providing energy ready to be used during strong physical activity. In addition, GC actively modulate the cardiovascular system, giving rise to elevations in heart rate, blood pressure and blood flow to the muscles. Also, GC suppress immunological function and decrease the inflammatory response, involving alterations in T-cell and B-cell function as well as in interlukin release (Griffin et al., 2014).

Naturally, although the short-term effects of GC are beneficial for the organism, pronounced and long lasting GC-induced changes in metabolic function can bring deleterious consequences. These include increased blood pressure, muscle tissue damage, steroid diabetes, infertility and inhibition of growth among others. Thus, in order to prevent such adverse effects, GC actions are tightly controlled by stringent negative feedback systems that modulate GC synthesis and secretion (Herman et al., 2012). Hence, after successfully coping with the stressor, the physiological profile is brought back to its resting state by binding of GC and CRH to different receptors in different brain regions (Wasserman et al., 2010). A key structure mediating this HPA axis regulation is the hippocampus, where GC receptors are expressed in pyramidal cells and dentate granule cells. In this regard, it has been shown that direct stimulation of this area can depress GC levels in rodents. Furthermore, hippocampal lesions blunt the HPA axis response to psychosocial stressors (Dranovsky and Leonardo, 2012). In addition, another important target to achieve robust modulation of the HPA axis response is the hypothalamus, where GC also exert strong regulatory effects by directly inhibiting CRH release and subsequent pituitary ACTH secretion (Pompili et al., 2010). Thus, GC negative feedback can take place directly on the hypothalamic cells driving the HPA axis.

Taken together, the different structures and peptides that comprise the HPA axis constitute key elements mediating the broad physiological, behavioral and functional alterations evoked by stress. Thus, any attempt to further elucidate the neurochemical candidates and molecular players responsible for the stress-induced cognitive dysfunctions must necessarily refer to its components. As previously mentioned, the CRH system is an essential element driving the activity of the HPA axis and thus plays a key role in the fast initiation of the stress response. Therefore, it has been considered as a prospective candidate mediating the myriad of cognitive and physiological effects caused by both, acute and chronic stress. Actually, alterations of the CRH system are implicated in different stress-related psychiatric disorders, mainly anxiety and depression (de Kloet et al., 2005; Holsboer and Ising, 2010). For this reason, it is fundamental to take a closer look at its different features in order to fully understand its physiology and its potential to become a therapeutic target to treat stress-related deficits.

II.5. The corticotropin-release hormone system

The CRH system comprises different ligands and receptor subtypes. In mammals, the main ligands are represented by CRH and Urocortins (UCN). Although they share about 45% sequence homology and bind to the same receptor family, they possess different affinities for the different receptor subtypes (Steckler and Holsboer, 1999). UCN is highly expressed in the Edinger- Westphal nucleus (Bonfiglio et al., 2011; Kozicz et al., 2011; Steckler and Holsboer, 1999). In rodents, there is a small overlap between CRH and UCN expression, giving support to the notion that both neuropeptides serve different functions. For example, previous studies have shown that, in contrast to CRH, UCN plays little or no role in the regulation and release of ACTH from the pituitary gland (Steckler and Holsboer, 1999).

II.5.1. CRH Expression

The CRH constitutes a 41-amino acid peptide hormone synthetized by the hypophysiotropic neurons of the PVN. The axons of these cells run laterally and caudally and terminate in the caudal zone of the medial eminence, where they release CRH into the portal vasculature of the anterior pituitary in order to initiate the stress response. In addition, cell clusters within the lateral, ventral and dorsal subdivisions of the PVN also synthetize CRH and project to preganglionic neurons of the medulla and spinal cord and

play a key role in the control of autonomic stress responses (Kovacs, 2013). Thus, hypothalamic CRH drives both basal and stress-induced HPA axis activation. However, the PVN does not constitute the only source of CRH in the brain. In fact, it has been shown that CRH is widely distributed across the central nervous system and the periphery, acting as both, neurotransmitter and neuromodulator (Merchenthaler, 1984; Palkovits et al., 1985). Among them, other CRH sources include the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BNST), which together comprise what is known as the extended amygdala. In addition, there are also CRH synthetizing neuron populations in the lateral hypothalamus, PFC, cingulate cortex, hippocampus, Barrington´s nucleus, parabrachial complex and nucleus of the solitary tract. Besides its role in the stress response, these CRH-containing neuronal populations have also been implicated in the regulation of arousal, executive functions, reward, fear, anxiety, depression, sleep-wake cycles, growth, reproduction, cardiovascular, metabolic and gastrointestinal functions. Therefore, abnormalities in extra-HPA axis CRH homeostasis have been associated with prevalent neuropsychiatric conditions including anxiety disorders, depression and Alzheimer disease, among others (Gallagher et al., 2008). Thus, whereas hypophyseotropic CRH drives the neuroendocrine stress cascade, extrahypothalamic CRH modulates the physiological and behavioral alterations necessary to prepare the organism for fight or flight, inhibiting as well vegetative and reproductive functions (Kovacs, 2013). In addition, it has been shown that during stress, central CRH also stimulates the locus coeruleus (LC), elevating noradrenaline and increasing arousal (Berridge and Waterhouse, 2003). Actually, intra-LC CRH microinjections mimic different sympathetic, behavioral and immune responses to stressors (Kubota et al., 2012).

II.5.2. CRH Regulation

Concerning the regulation of CRH transcription within the aforementioned circuits, it is known that it can be tightly modulated in a site-, stress- and GC-dependent manner. For instance, it has been established that acute stress challenges lead to significant increases in CRH mRNA and peptide levels in hypothalamic and extra-hypothalamic regions (Kovacs and Sawchenko, 1996) together with significant reductions in the olfactory bulb (Imaki et al., 1991). Along this line, different studies have also demonstrated immediate robust increases in CRH release from the PVN, with a concomitant depletion of neuropeptide stores at the axon terminals, following acute stress exposure (Plotsky, 1985). Basically, once a situation is perceived as potentially aversive, stress-related afferents activate different signal transduction pathways in the PVN neurosecretory cells that converge upon the

regulatory region of the CRH gene in order to initiate CRH transcription. In this regard, it has been shown that changes in PVN CRH mRNA levels depend on the integrity of ascending catecholaminergic (noradrenergic) projections originating in the brain stem (Pacak et al., 1996). In fact, stress-induced effects on CRH synthesis can be mimicked by intra-PVN norepinephrine microinjections (Cole and Sawchenko, 2002; Itoi et al., 1999). However, this process seems to be far from simple and to require the recruitment of specific coactivators such as the transducer of Regulated CREB activity and the interactions with several trans-acting factors like the inducible cAMP early repressor (Martin et al., 2012; Watts et al., 2011). Interestingly, whereas glutamatergic neurotransmission doesn´t seem to play a role in the regulation of CRH transcription, interference with the GABAergic inputs to the PVN increase CRH expression in this structure (Cole and Sawchenko, 2002).

Besides the PVN, stress also induces CRH upregulation in several extrahypothalamic regions, including limbic areas such as CeA, BNST and Barrington´s nucleus (Hsu et al., 1998; Makino et al., 1999), which seem to be independent of brainstem ascending catecholaminergic afferents (Pacak et al., 1996). Furthermore, several lines of evidence document the role of corticosterone in the regulation of the amygdalar CRH expression (Schulkin et al., 1998; Watts, 2005). Given such findings, it has been proposed that CeA CRH may play a key role in the modulation of HPA axis activity under stress. Actually, lentiviral-induced CRH overexpression in the CeA evokes increases in CRH and Arginin Vasopressin (AVP) expression in the PVN and impairs GC negative feedback on ACTH secretion (Keen-Rhinehart et al., 2009). In addition, different stress paradigms and CRH administration similarly increase CRH expression in several cortical and limbic areas such as PFC, cingulate cortex and hippocampus (Givalois et al., 2000).

Once stress triggers the activity of the HPA axis through the synthesis and release of CRH, it is also fundamental to be able to effectively constrain this physiological cascade in order to avoid the many deleterious effects of long-term GC exposure. A key mechanism subserving this regulatory action relies on the negative feedback mediated by GCs in the hypophysiotropic CRH expressing neurons within the PVN, through a putative negative GC response element located on the CRH gene. Thus, these CRH-expressing PVN neurons have been shown to be particularly sensitive to GCs (Bali et al., 2008; Kovacs et al., 2000). In fact, it has been shown that the targeted disruption of GR signaling within the PVN results in the upregulation of CRH mRNA levels (Jeanneteau et al., 2012a).

Additionally, GCs also affect the half-life of the CRH transcripts. For instance, it is known that adrenalectomy decreases the rate of CRH mRNA degradation and corticosterone replacement diminishes the half-life of CRH mRNA in the rat PVN (Ma et al., 2001).

II.5.3. CRH Receptors

Once released, CRH induces all of its physiological and cellular effects through the activation of different receptors. Mainly, CRH acts on two different kinds of G-protein coupled receptors (GPCR): CRHR1 and CRHR2, both exhibiting a 70% sequence homology and also possessing molecular splice variants (Perrin and Vale, 1999), including CRHR1α, CRHR1β and $CRHR_{c\text{-}n}$ for CRHR1. On the other hand, CRHR2 has three different isoforms, CRHR2α, CRHR2β and CRHR2γ, the last one being only described in humans (Bonfiglio et al., 2011). Both receptor subtypes also differ in the affinities expressed by their ligands and display distinct pharmacological profiles (Dautzenberg and Hauger, 2002; Fekete and Zorrilla, 2007). Both CRHRs constitute α-helical GPCR with 7-transmembrane domains, possessing an N-terminal end on the extracellular side and a C-terminus facing the cytoplasm of the cell. On the extracellular side, they possess a binding domain for CRH. On the intracellular side, they express a guanyl-nucleotide exchange factor (GEF) protein that is activated upon the binding of their ligand to the extracellular binding domain (Bonfiglio et al., 2011; Steckler and Holsboer, 1999).

Despite the fact that initially it was believed that CRHR1 expression was exclusively restricted to the HPA axis, several lines of evidence have demonstrated that in fact they are widely expressed across the neuroaxis. CRHR1 immunoreactivity has been shown in cholinergic, dopaminergic and noradrenergic neurons of the murine basal forebrain and brainstem nuclei (Sauvage and Steckler, 2001). More specifically, CRHR1 is highly expressed in the anterior pituitary, neocortical areas, basolateral and medial nuclei of the amygdala, hypothalamic nuclei, cerebellar purkinje cells, lateral dorsal tegmentum, pedunculopontine tegmental nucleus and PFC. On the other hand, CRHR2 distribution exhibits a more discrete pattern, comprising the lateral septum, ventromedial hypothalamus and cortical nucleus of the amygdala (Bonfiglio et al., 2011). Foremost, it has been suggested that each CRHR exerts a synapse specific depression or facilitation of excitatory transmission. For instance, at the basolateral amygdala-central amygdala synapse, activation of postsynaptic CRHR1 results in a net depression of evoked glutamatergic neurotransmission despite weak pre, and postsynaptic, facilitatory actions mediated by CRHR2 stimulation (Liu et al., 2004).

The differences in CRHRs expression have given rise to the idea that both receptor types subserve different functions in stress-related processes. In fact, several animal studies have suggested that the behavioral and hormonal effects of CRH can be ascribed to CRHR1-mediated actions (Smith et al., 1998; Timpl et al., 1998). Also, CRHR1 has been identified as the main mediator of the stress-induced neuroendocrine, physiological and behavioral CRH actions (Holsboer, 1999). Consequently, CRHR1-null mice exhibit reduced anxiety-related behavior and impaired basal and stress-induced HPA axis response. Furthermore, limbic CRHR1 has been proposed to be strongly involved in the feedback regulation of the HPA axis (Muller et al., 2003). In addition, it is known that during acute stress, CRH is rapidly released in the synapses, facilitating hippocampal functions through increases in synaptic plasticity and long-term potentiation (LTP) via postsynaptic CRHR1s (Chen et al., 2004). Similarly, CRHR1 is responsible for the CRH-induced effects in noradrenergic neurotransmission and arousal (Jedema and Grace, 2004). Moreover, specific CRHR1 deletion in limbic structures gives rise to hypersensitivity to stress and decreases in anxiety-like behavior (Refojo et al., 2011a).

In agreement with the findings already mentioned, CRH overexpression targeted to different brain regions has been shown to induce HPA axis abnormalities, increases in rapid-eye movement (REM) sleep, anxiogenesis, increased acustic startle and passive stress coping behavior (Flandreau et al., 2012; Groenink et al., 2002; Kimura et al., 2010; Kolber et al., 2010). Furthermore, silencing CRH expression in the CeA of adult mice decreases stress-induced anxiety-like behaviors and gives rise to increases in basal corticosterone (CORT) levels (Regev et al., 2012).

As already mentioned, CRHR1 constitutes the main effector candidate through which CRH initiates the stress response and induces many of its actions. Thus, it is essential to fully understand the molecular pathways activated by the stimulation of this receptor, in order to further identify potential mechanisms responsible for the behavioral alterations evoked by environmental stressors. Such knowledge will surely draw attention towards prospective therapeutic targets that eventually will aid in the alleviation of different stress-related disorders.

II.6. CRHR1-mediated signaling pathways

CRHR1 displays a wide distribution pattern across the neuraxis. Accordingly, several lines of evidence have established that CRHR1 can activate different signaling pathways in a region-specific and activity-dependent manner. CRHR1 exhibits a number of diverse splicing variants (α, ß, c-n) that also contribute to region-specific differences in receptor-signaling properties. However, CRHR1α seems to be the predominantly expressed and functional form in rodents and humans. In most systems, ligand-activated CRHR1 signals through Gα coupling, leading to the stimulation of adenylyl cyclase. This is followed by increases in the generation of the second messenger cyclic Adenosine Monophosphate (cAMP) from Adenosine Triphosphate (ATP), which in turn activates protein kinase A (PKA). PKA comprises a functional tetramer composed by two catalytic subunits (PKA-C) and two regulatory subunits (PKA-R). These PKA-R regulate the activity of the PKA-C and are also responsible for binding specific A kinase-anchoring proteins (AKAPs). By limiting PKAs to certain cellular compartments, these AKAPs are key elements mediating specific subcellular compartmental signaling. For instance, dynamic conditions can lead to changes in the localization of AKAPs, thus affecting the localization and activation of the PKA (Taylor et al., 2005; Taylor et al., 2012). In its inactive state, the four components are linked together. However, once cAMP is produced by adenylyl cyclase after Gα stimulation, cAMP binds to the PKA-Rs, inducing a conformational change and thus releasing the PKA-Cs. These PKA-Cs are then translocated to the nucleus, where they phosphorylate candidates downstream of cytosolic and nuclear targets, such as the cAMP responsive element binding protein (CREB), consequently affecting genetic transcription (Figure 4). Particularly in the pituitary, a crosstalk between this pathway and PKC (via activation of the V1b subtype vasopressin receptor) seems to be responsible for the increases in ACTH synthesis and release observed during stress (Bonfiglio et al., 2011).

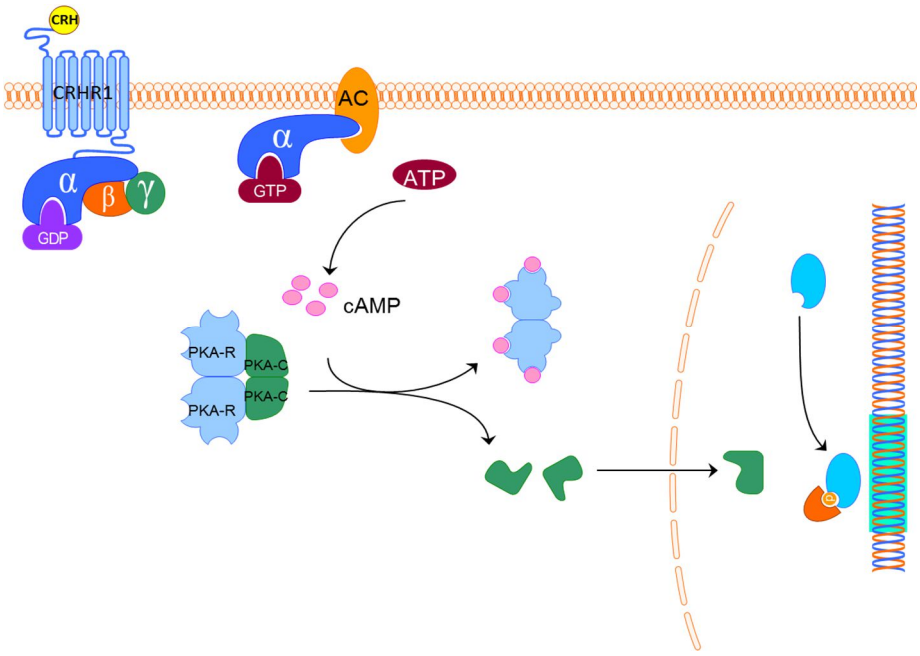

Figure 4. PKA signaling pathway
Once the ligand CRH binds to the CRHR1 receptor, it induces an exchange between Guanosine Diphosphate (GDP) and Guanosine Trisphosphate (GTP), which leads to the translocation of the Gα subunit and the activation of Adenylyl Cyclase (AC). Posteriorly, AC catalyzes the conversion of Adenosine Triphosphate (ATP) into 3"-5" cyclic Adenosine Monophosphate (cAMP), which binds to the regulatory subunit of Protein Kinase A (PKA-R) and induces the release of its catalytic subunit (PKA-C) and its translocation to the nucleus. Consequently, it phosphorylates the transcription factor cAMP Response Element Binding Protein (CREB), promoting genetic transcription.

The PKA-dependent signaling pathways have been shown to play a fundamental role in diverse memory functions, including working, short-term and long-term memory (Kobori et al., 2015a). In addition, it has also been demonstrated to regulate different neurophysiological processes, such as LTP, and thus has a key role in neuronal plasticity (Marsden, 2013). Furthermore, PKA signaling is linked to age-related cognitive alterations, particularly concerning working memory (Ramos et al., 2003).

Besides the classical Gs activation leading to adenylyl cyclase stimulation, different reports in the literature have shown that CRHR1 is also linked to other intracellular signaling pathways, being able also to interact with G_q, G_i, G_o, $G_{i1/2}$ and G_z proteins in order to transmit signals to diverse effectors in an agonist and cell-specific manner (Dautzenberg and Hauger, 2002; Grammatopoulos et al., 2001). Thus, CRHR1 can modulate several signaling pathways and kinases besides PKA, including PKB, PKC, mitogen activated protein kinases (MAPK) and intracellular Ca^{2+} concentrations in a tissue-specific and

concentration-dependent manner (Grammatopoulos and Chrousos, 2002). The activation of MAPKs cascades by CRHR1 involves the participation of different signaling mediators (Brar et al., 2004). For instance, mechanisms including heterotrimeric G-Proteins, small G-proteins of the Ras family and G-protein-independent activation via ß-arrestin-mediated receptor desensitization have been described, depending on the system analyzed. Furthermore, UCNI, another CRHR1 endogenous agonist, has been shown to couple CRHR1 to Gq/phospholipase C/IP3/PKC pathways. In other words, CRHR1 may actually activate diverse mechanisms in the same cellular context, exhibiting different spatiotemporal characteristics of MAPK activation (Arzt and Holsboer, 2006; Lefkowitz and Shenoy, 2005).

In the pituitary, studies on CRH-induced signaling, via the activation of CRHR1, have delineated the different MAPK signal transduction pathways activated, downstream of cAMP/PKA, that eventually lead to the modulation of transcription factors that regulate POMC expression (Timpl et al., 1998). Thus, using AtT-20 corticotrophs, CRH was shown to activate the transcription factors CREB, c-fos and the orphan receptor Nur77 through PKA (Hauger et al., 2006). Basically, PKA triggers mainly two transduction pathways: one calcium-independent and another dependent on calcium influx through voltage-dependent calcium channels located at the plasma membrane, which involve calmodulin kinase II. In addition, the extracellular signal regulated kinase 1/2 (ERK1/2) is also activated by CRH via CRHR1 in corticotrophs, involving the activity of small G-protein Rap1, the B-Raf kinase. ERK1/2 regulates the induction and activity of the target transcription factors that control POMC transcription in the pituitary (Kovalovsky et al., 2002).

However, CRHR1-mediated signal transduction seems to depend also on cellular context. For instance, it is known that cell lines resembling noradrenergic locus coeruleus neurons activate ERK1/2 via CRHR1 and cAMP/PKA (Cibelli et al., 2001). On the other hand, HEK293 cells expressing CRHR1 trigger cAMP through the activation of Gαs without activating ERK1/2 (Grammatopoulos et al., 2000). Foremost, increases in ERK2 phosphorylation within the PFC have been observed after acute restraint stress (Meller et al., 2003). Interestingly, cAMP has been shown to have stimulatory and inhibitory effects on ERK1/2 activation depending on cellular context. Furthermore, additional studies have demonstrated that CRHR1-dependent activation of ERK1/2 phosphorylates numerous proteins along the limbic system, playing also a key role in memory, learning, and neuroplasticity (Refojo et al., 2005; Thomas and Huganir, 2004). Surprisingly, CRH-

mediated ERK1/2 activation displays high region specificity, as different hippocampal and amygdalar areas exhibit differential activation patterns upon CRH administration (Refojo et al., 2005). Taken together, these findings suggest that CRH activates, via the same receptor, diverse pathways in different cell types depending as well on the intracellular context (Arzt and Holsboer, 2006). However, information concerning CRHR1-dependent signaling on the PFC is still lacking.

II.7. Aim

The main aim of the present thesis was to establish the role played by the CRH-CRHR1 system in the acute stress-induced executive dysfunction via PKA. In order to achieve this goal, the project was subsequently subdivided into different specific aims:

1. Determine the impact of acute stress in PFC-mediated cognition.
2. Identify the role played by the CRHR1 on the impact of acute stress in PFC-mediated cognition.
3. Establish the effects of the intra-PFC CRH microinjection in PFC-mediated cognition.
4. Determine the effects of acute stress and intra-PFC CRH microinjection in PKA activity.
5. Identify the role of PKA on the impact of the intra-PFC CRH microinjection in PFC-mediated cognition.

III. MATERIALS AND METHODS

III.1. Animals

The present experiments were performed using 12 weeks old male C57Bl6 mice, purchased from Charles River (Charles River Laboratories, France). In addition, mice expressing conditional CRHR1 alleles ($CRHR1^{lox/lox}$) with 12 weeks of age were used to study the effects of CRHR1 deletion on the acute stress-induced PFC-dysfunction. As well, CRFcre x Ai9 x R1GFP mice were used to determine the expression of CRH and CRHR1 within PFC networks. Furthermore, CD1 mice were employed as aggressors during the acute social defeat paradigm. All rodents were single housed in polycarbonate cages (21 x 15 x 14cm), using standard bedding and nesting material, under a 12 hour light-dark cycle (lights on at 7am) with constant temperature (23 ± 2°C) and humidity (55 ± 5%). Animals had free access to standard mouse chow (Altromin 1324, Altromin GmbH, Germany) and tap water. All experimental procedures were carried out in the animal facility of the Max Planck Institute of Psychiatry in Munich, Germany, in accordance with the European Communities Council Directive 2010/63/EU. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

III.2. Experimental Design

III.2.1. Experiment 1: Effects of acute stress in PFC-mediated cognition

III.2.1.1. Effects of acute stress in temporal order memory

To identify the effects of acute stress in temporal order memory, 23 C57Bl6 mice were employed. Once the animals arrived at the animal facility of the Max Planck Institute of Psychiatry, they remained undisturbed for 7 days in order to allow them to familiarize with the new environment. After this period, they were submitted to the habituation phase of the temporal order memory test, described in detail in the section III.4.1. After this, they were submitted to a single episode of acute social defeat stress (n=12), described in detail in the section III.3, or to control conditions (n=11). Basically, the mice were placed in the homecage of a CD1 resident for a period of 5 minutes, during which they engaged in agonistic confrontations. After this, the experimental animals were returned to their

respective homecages and 5.5 hours later were submitted to the temporal order memory test. On sample phase I, animals were placed in the experimental apparatus containing two identical objects for 10 minutes. After 1 hour, they underwent the sample phase II, in which they were again placed in the same environment with two identical copies of a different object for 10 minutes. 1 hour later, the test phase took place by placing the rodents in the same context, containing one copy of the object used in sample phase I (Old object) and one copy of the object used in sample phase II (Recent object). The animals were allowed to explore both objects for 10 minutes and the time spent exploring each object was scored by an observer.

III.2.1.2. Effects of acute stress in reversal learning

The aim of this study was to establish the effects of acute stress in reversal learning. To accomplish this, 20 C57Bl6 mice were used. After their arrival at the animal facility of the Max Planck Institute of Psychiatry, the animals were single-housed and remained undisturbed in their homecage for a period of 7 days, in order to allow them to familiarize with the new housing conditions. Then, they were submitted to the reversal learning test as described in the section III.4.2. During the 3rd day, animals were submitted to a single episode of social defeat stress (n=11) or to control conditions (n=9), and 6 hours later underwent the reversal session. The number of mistakes made before the first correct response, together with the total trials to reach criteria were scored by the experimenter.

III.2.2. Experiment 2: Visualization of the CRH- and CRHR1-containing neural circuits within the PFC networks

Aiming to visualize the CRH- and CRHR1-containing neural populations potentially responsible for the acute stress-induced PFC dysfunction, PFC tissue of a CRFcre x Ai9 x R1GFP mouse was submitted to the clarity protocol as described in detail in the section III.7. Briefly, the mouse was intracardially perfused and the PFC was extracted and incubated in Hydrogel solution. After 3 days, it was submitted to a clearing process and then to an immunofluorescence protocol against GFP. Posteriorly, sample overview and single cell images were acquired using LaVision Light Sheet software and an Olympus Confocal microscope respectively.

III.2.3. Experiment 3: Effects of acute stress on CRHR1 mRNA expression in the PFC

In order to determine the role of acute stress in CRHR1 mRNA expression within the PFC, a total of 24 C57Bl6 mice were employed. Animals were submitted to a single episode of acute social defeat stress (n=12) or to control conditions (n=12), and 8 hours later were sacrificed. Their brains were extracted and frozen at -80°C. Posteriorly, coronal sections of these brains, containing the mPFC, were submitted to an *In Situ* Hybridization protocol to detect CRHR1 mRNA levels (See III.6.1).

III.2.4. Experiment 4: Effects of intra-PFC CRHR1 deletion on the acute stress-induced executive dysfunction

III.2.4.1. Effects of intra-PFC CRHR1 deletion on the impact of acute stress in temporal order memory

The goal of this experiment was to study the involvement of the intra-PFC CRHR1 on the impairment in temporal order memory caused by acute stress. With this purpose, 37 CRHR1$^{lox/lox}$ mice were submitted to stereotaxic surgery (described in detail in the section III.5.1) and received intra-PFC microinjections of an Adeno Associated Virus (AAV) containing either Cre or empty expression cassettes. After surgery, they remained in the animal facility for 30 days in order to allow a full expression of the transferred gene, leading to a CRHR1-CKO (Cre)(n=20) or -WT (Empty)(n=17) exclusively in the PFC. At the beginning, animals underwent the habituation phase of the temporal order memory. On the 7th day, rodents were subdivided in 4 groups and submitted to either control conditions (PFC CRHR1-CKO, n=10; WT, n=8), or to an acute social defeat (PFC CRHR1-CKO, n=10; WT, n=9), and 5.5 hours later underwent the temporal order memory test.

III.2.4.2. Effects of intra-PFC CRHR1 deletion on the impact of acute stress in reversal learning

To further delineate the role played by the intra-PFC CRHR1 in the acute stress-induced deficits in PFC-mediated cognition, the mice used in the previous experiment were also employed to assess the effects of the intra-PFC CRHR1 deletion on the impact of acute stress in reversal learning. Once finished the temporal order memory test, the animals were left undisturbed for one week to allow them to recover from the experimental sessions.

Posteriorly, on the following 2 days, they were submitted to the training and retention sessions of the reversal learning test. Based on their performance, 4 animals had to be discarded for not reaching criteria in less than 30 trials. On the 3rd day, they were again subdivided in the same groups as before: control WT (n=7), control PFC CRHR1-CKO (n=9), stressed WT (n=7) and stressed PFC CRHR1-CKO (n=10), and submitted to either acute social defeat stress, or control conditions accordingly. After 6 hours, all rodents underwent the reversal session of the test. In order to confirm the successful deletion of the CRHR1, all animals were sacrificed after the experiment. Their brains were extracted and kept at -80°C. Coronal sections of these brains containing the PFC were then submitted to a CRHR1 autoradiography (See III.6.2 for more details) and the amount of CRHR1 binding was calculated for each group of animals.

III.2.5. Experiment 5: Effects of intra-PFC CRH microinjections in PFC-mediated cognition

III.2.5.1. Effects of intra-PFC CRH microinjections in temporal order memory

This study was performed with the aim of establishing the effects of intra-PFC CRH microinjections in temporal order memory. To achieve this goal, 20 C57Bl6 mice were submitted to stereotaxic surgery and a guide cannula was implanted in the PFC. After the surgery, the animals were allowed to recover from the procedure during 1 week. Concluded this period, they underwent the habituation protocol for 7 days. The last day, rodents received intra-PFC microinjections of either ACSF (Vehicle, n=10) or CRH (0.6mM)(n=10), and 5.5 hours later were submitted to the temporal order memory test. Once finished the experimental procedure, the mice were sacrificed and their brains were extracted and frozen at -80°C. Coronal sections of these animals were then stained with cresyl violet to confirm the microinjection sites. Animals with microinjection points outside the PFC were discarded from the study.

III.2.5.2. Effects of intra-PFC CRH microinjections in reversal learning

In order to elucidate the effects of intra-PFC CRH microinjections in reversal learning, 21 C57Bl6 mice were submitted to stereotaxic surgery and a guide cannula was implanted in the PFC. The animals were left undisturbed for 1 week and posteriorly underwent the training and retention sessions of the reversal learning test. On the 3rd day, they received intra-PFC micronjections of either ACSF (n=10) or CRH (0.6mM)(n=11), and 6 hours

later were submitted to the reversal phase of the reversal learning test. After the experimental procedure, the mice were sacrificed and their brains were extracted and frozen at -80°C. Coronal sections of these animals were then stained with cresyl violet to confirm the microinjection sites. Animals with microinjection points outside the PFC were discarded from the study.

III.2.6. Experiment 6: Effects of CRHR1 blockade on the intra-PFC CRH-induced executive dysfunction

III.2.6.1. Effects of CRHR1 blockade on the intra-PFC CRH-induced deficits in temporal order memory

The goal of the present experiment was to determine the effects of the CRHR1 blockade on the impact of intra-PFC CRH in temporal order memory. With this aim, 35 C57Bl6 mice were submitted to stereotaxic surgery and a guide cannula was implanted in the PFC. The animals were allowed to recover from the procedure for 1 week and afterwards underwent the habituation phase of the temporal order memory test. Posteriorly, they received intra-PFC microinjections of ACSF (n=9), the CRHR antagonist Astressin (1μM)(n=8), CRH (0.1 μM)(n=10) or a combination of Astressin + CRH (n=8), and 5.5 hours later were submitted to the temporal order memory test.

III.2.6.2. Effects of CRHR1 blockade on the intra-PFC CRH-induced deficits in reversal learning

To further explore the role of the CRHR1 in the CRH-induced deficits on PFC-mediated cognition, 33 C57Bl6 mice with a cannula implanted in the PFC were employed. They were submitted to the training and retention sessions of the reversal learning test and on the 3rd day received intra-PFC microinjections of ACSF (n=8), the CRHR antagonist Astressin (1μM)(n=8), CRH (n=9) or a combination of Astressin + CRH (0.1 μM)(n=8), and 6 hours later underwent the reversal phase of the test. Once finished the experimental procedure, the mice were sacrificed and their brains were extracted and frozen at -80°C. Coronal sections of these animals were then stained with cresyl violet to confirm the microinjection sites. Animals with microinjection points outside the PFC were discarded from the study.

III.2.7. Experiment 7: Effects of acute stress in the activity of protein kinase A

This study was made with the aim of establishing the effects of acute stress on the activity of the protein kinase A. To achieve this, 16 C57Bl6 mice were submitted to a single episode of acute social defeat stress or control conditions and 15 minutes later were sacrificed. The PFC was dissected and further processed in order to perform a Co-immunoprecipitation to detect PKA-R binding to PKA-C and PKA-C binding to CREB (For details see III.6.4). In addition, samples of these lysates were subsequently submitted to a western blot protocol to analyze pCREB levels.

III.2.8. Experiment 8: Effects of intra-PFC CRH microinjections in the activity of protein kinase A

In order to determine the effects of intra-PFC CRH in the activity of PKA, 13 C57Bl6 mice were used. They were submitted to stereotaxic surgery and a guide cannula was implanted in the PFC. The animals were allowed to recover from the surgery for 1 week. Afterwards, they received intra-PFC microinjections of ACSF (n=6) or CRH (n=7), and 15 minutes later were sacrificed. Their PFC was dissected and further processed in order to perform a Co-immunoprecipitation to detect PKA-R binding to PKA-C and PKA-C binding to CREB. In addition, samples of these lysates were subsequently submitted to a western blot protocol to analyze pCREB levels.

III.2.9. Experiment 9: Effects of PKA blockade on the intra-PFC CRH-induced executive dysfunction.

III.2.9.1. Effects of PKA blockade on the intra-PFC CRH-induced deficits in temporal order memory

The goal of the present experiment was to clarify the effects of PKA blockade on the intra-PFC CRH-induced deficits in temporal order memory. To achieve this, 47 C57Bl6 mice were submitted to stereotaxic surgery and a guide cannula was implanted in the PFC. The animals were left undisturbed for 1 week after surgery. Afterwards, they were submitted to the habituation period of the temporal order memory test. On the 7th day, they received

intra-PFC microinjections of ACSF (n=12), the PKA inhibitor Rp-cAMPS (n=12), CRH (n=11) or a combination of Rp-cAMPS + CRH (n=12), and 5.5 hours after the microinjections underwent the temporal order memory test.

III.2.9.2. Effects of PKA blockade on the intra-PFC CRH-induced deficits in reversal learning

To further establish the role of PKA on the acute stress-induced, CRHR1-dependent, PFC dysfunction, 48 C57Bl6 mice with a cannula implanted in the PFC were used. They were submitted to the training and retention sessions of the reversal learning test. On the 3rd day, they received intra-PFC microinjections of ACSF (n=12), Rp-cAMPS (n=10), CRH (n=14) or a combination of Rp-cAMPS + CRH (n=12) and 6 hours later underwent the reversal session. After the experiment, the mice were sacrificed and their brains were extracted and frozen at -80°C. Coronal sections of these brains were then stained with cresyl violet to confirm the microinjection sites. Animals with microinjection points outside the PFC were discarded from the study.

III.3. Acute Social Defeat Stress Paradigm

Aggressive interactions between conspecifics can trigger the stress response and correspondingly activate the HPA axis. Therefore, the facilitation of aversive encounters constitutes a naturalistic approach that mimics threatening scenarios encountered in daily life. Hence, the acute social defeat stress paradigm was employed as a natural procedure to induce a stress reaction in rodents. It was performed as described by Wagner et al., 2013. CD1 mice were allowed to habituate to the social defeat cage for a period of 2 weeks before the experiments. Throughout this time, their latency to attack was measured every three days, by placing a novel C57Bl6 rodent in the defeat cage. Only those CD1 residents with latencies shorter than 5 seconds were selected for the experiments. During the acute social defeat stress, experimental animals were placed in the CD1 homecage and allowed to engage in agonistic confrontations with the CD1 resident for 5 minutes. The experimenter only interfered with the aggressive encounters in case of severe injury. In such cases, the procedure was stopped and the experimental animal was discarded from the study. Control animals were allowed to explore an empty novel cage similar to the defeat cage for 5 minutes.

III.4. Behavioral tests

III.4.1. Temporal Order Memory Test

The temporal order memory test constitutes a procedure designed to asses prefrontal cortex mediated episodic-like memory in mice (Barker et al., 2007). Basically, it demands recognition memory processes, requiring judgements about the previous occurrence of events through the use of recency information. Initially, in order to guarantee high exploratory rates of the different objects employed during the test, mice were submitted to a thorough habituation procedure. They were handled by the experimenter for 2 minutes during 7 consecutive days, in order to familiarize them to the experimenter and to the manipulation that takes place during the test. On the 3rd day of handling, the mice were habituated to the apparatus, consisting of a type III rodent cage 38.2 cm length X 22 cm wide X 15 cm high with a thin layer of clean sawdust. They were placed in this cage for 2 minutes during 2 consecutive days. After this, in order to decrease neophobia, a salt shaker was left overnight in their homecage. The next day, animals were familiarized to the presence of objects on the apparatus and the time schedule of the test. For this, 2 identical copies of an object, different from the one used during the test, were placed in opposite sides of the apparatus. The rodents were then submitted to 3 trials, each of 10 minutes, with an inter-trial interval of 60 minutes. During each trial, the mice were placed in the center of the open field and allowed to explore the objects for a period of 10 minutes.

Once concluded the habituation period, the animals underwent the temporal order memory test, composed by 3 different trials of 10 minutes each. The objects employed in this test correspond to the set of objects used by Knappman et al., 2010 built out of lego blocks (Figure 5A). The next day after the last habituation session, during the sample phase I, 2 identical copies of an object were placed in opposite sides of the cage. Each mouse was located in the center of the apparatus and allowed to explore the objects for 10 minutes. Then, it was removed from the cage and taken back to its home cage. After 1 hour, the sample phase II took place. For this, 2 identical copies of a different object were again placed in opposite sides of the apparatus and the animal was allowed to explore them for 10 minutes. Finished this period, the mouse was left in its home cage for another hour until the test phase. Then, a copy of the object used in sample phase I (Old) and a copy of the object used in sample phase II (Recent) were used. Both objects were located in opposite sides of the cage and the animal was allowed to explore them for 10 minutes (Figure 5B). The objects were counterbalanced between groups in order to avoid any bias caused by a

preference towards any of the objects. The time spent exploring each of the objects was scored. An animal with intact temporal order memory shows a preference for the old object, exhibiting a higher exploration time of this object when compared with the time spent exploring the recent object. The results are expressed in 2 different ways. On one hand, the percentage of exploration for each object is shown for each group of animals. In addition, another analysis is employed using a discrimination index between both objects that is calculated as follows:

$$\text{DI } (\%) = 100 \times \frac{Time\ with\ the\ old\ object - Time\ with\ the\ recent\ object}{Total\ time\ with\ both\ objects}$$

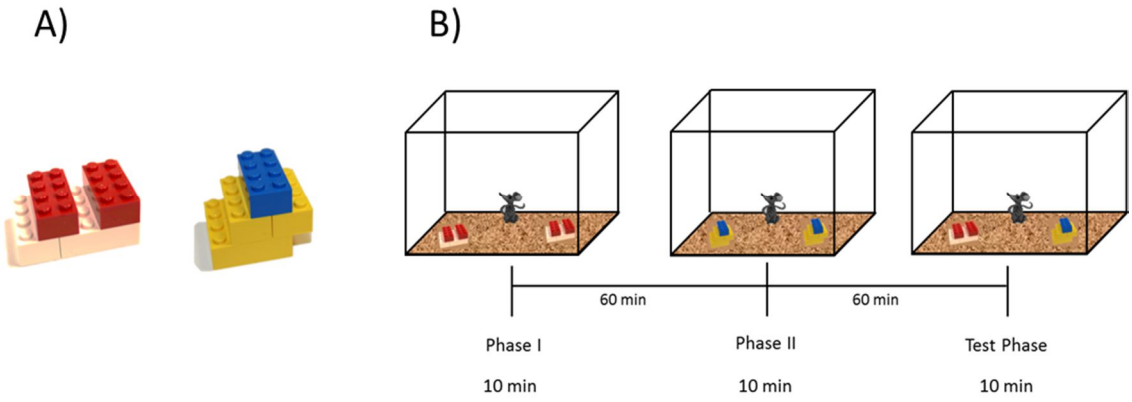

Figure 5 Temporal Order Memory Test
A) Sample pictures of the objects employed on the temporal order memory test (Knapman et al., 2010). B) Schematic diagram depicting the different phases and time schedules that compose the temporal order memory test.

III.4.2. Reversal Learning Test

The reversal learning test constitutes a task employed to assess cognitive flexibility, defined as the ability to switch a predominant behavioral response for an opposite pattern, according to environmental feedback. This cognitive process has been shown to depend as well on the integrity of the PFC networks, and was used in the present study to determine the effects of stress, via the CRH-CRHR1 system, on PFC-related functions (Clark et al., 2004). This test was performed as described by Knappman et al., 2010. It is subdivided in 3 different sessions, distributed across 3 days. The experimental setup consists on a T-maze with 3 50cm long arms made of Plexiglass, mounted on a 50cm high table. The arms could be opened or closed individually using transparent Plexiglas pulley doors. In order to allow

the animals a clear distinction between each of the arms, their walls were covered with different color papers as follows: The start arm was covered in black paper with diagonal white stripes; one of the goal arms was covered in white paper and the opposite goal arm was covered in black. One of the goal arms was connected to an escape wire tunnel with a diameter of 5 cm. This tunnel led back to the homecage of the mouse, located on the floor to avoid any visual or olfactory cues at the center of the maze. The opposite goal arm was connected to a "dummy" tunnel, also made of chicken wire, with a dead end after 20 cm. This ensured that both arms looked identical from the center of the maze, where the mouse decides which arm to take.

On the training session, the animal was placed at the beginning of the start arm. Once the rodent arrived at the center zone, the pulley door from the start arm was closed, forcing the animal to choose one of the goal arms. After entering one of the goal arms, the corresponding pulley door was closed. If the mouse entered the correct arm, it was allowed to reach its homecage via the escape tunnel, which served as a reinforcer in itself. If the animal took the incorrect arm, once it entered the "dummy" tunnel it received a mildly aversive air-puff and was left in the tunnel for 30 sec. Afterwards, it was immediately placed back in its homecage. With this procedure, the rodents were trained to take the correct arm. The arm containing the escape tunnel was counterbalanced within groups to avoid a bias caused by a preference towards a specific arm. During each session multiple trials were performed, with an inter-trial interval of 5 min, until the mice reached 8 correct choices out of 10 consecutive trials. Once this learning criteria was achieved, the animal was left undisturbed in his homecage until the next day. If a rodent did not fulfill criteria before 30 trials, it was discarded from the study.

Given the short inter-trial interval, the rodents were retested 24 hours later to ensure a robust consolidation of the behavioral response. During this retention session, the same procedure was repeated until the mice reached criteria. On the 3rd day, the correct and incorrect arms were switched. Thus, during the reversal session, animals had to learn to take the opposite arm in order to reach their homecage (Figure 6). The same learning criteria was applied and scored. In addition, the number of perseverative errors, or mistakes made before the first correct response was also registered.

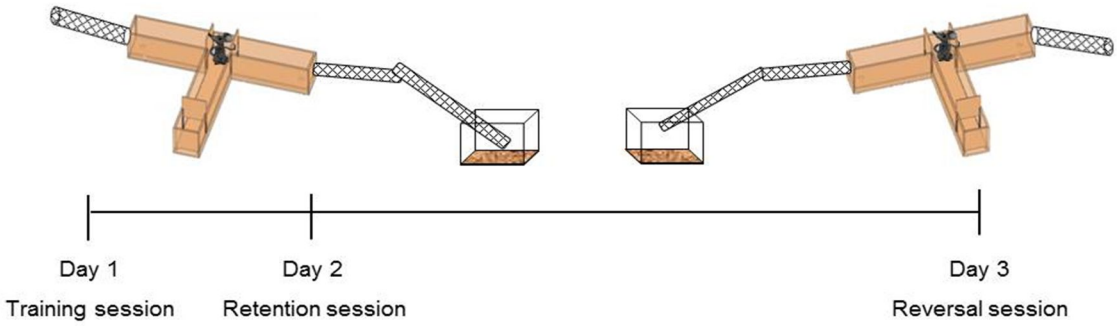

Figure 6 Schematic diagram of the experimental design of the reversal learning test.
The reversal learning test is subdivided into three different sessions, performed along three consecutive days: Training session, retention session and reversal session. During the training session, animals are trained to take the arm leading to their homecage. On the retention session, the same procedure is employed to ensure that the animals have consolidated the behavioral task. On the reversal session, the goal and dummy arms are exchanged and the animals must learn to take the opposite arm in order to reach their homecage (Knapman et al., 2010).

III.5. Intra-PFC microinjections

III.5.1. Stereotaxic Surgery

In order to perform intra-PFC microinjections according to the different experimental paradigms employed, mice were submitted to stereotaxic surgery. To begin, animals were shortly anesthesized with isoflurane. Immediately after, they were quickly placed under 2% isoflurane anaesthesia with a mask and fixated on the stereotaxic frame through the incisive and ear bars. Additionally, a heating pad was placed under the rodent to prevent a decrease in body temperature. Mice were then subcutaneously injected with 200 µl of metacam (concentration: 62.5 mg/ml) to increase analgesia. The head of the animals was trimmed and treated with iodine while the eyes were covered with ophthalmic ointment. An incision was performed on the skin above the skull using a scalpel and the subcutaneous tissue was moved aside. The surface of the skull was cleaned with oxygenated water in order to facilitate the visualization of the joints between the different bones. The coordinates of bregma and lambda were determined under the binocular. All microinjections were targeted at the PFC, encompassing Prelimbic and Infralimbic regions. The holes for unilateral cannula implantation/ bilateral AAV microinjections were drilled with a 0.6 mm drillhead using the following coordinates from bregma: anterior/posterior -1,94mm anterior to bregma and mediolateral, +0,4mm from the midline.

III.5.2. Intra-PFC AAV microinjection

The glass micropipettes employed for Adeno Associated Virus (AAV) microinjection were pulled from glass capillaries. The tip of the micropipette was cut off, and it was filled with 0.5 μl of the corresponding AAV (Empty or Cre). For the viral-induced CRHR1 deletion, an AAV1/2 vector from GeneDetect (New Zealand) containing CAG-HA-NLS-Cre-recombinase-WPRE-BGH-polyA was employed. The same vector was used for the control groups (Empty group) containing the cassette CAG-Null/Empty-WPRE-BGH-polyA. The glass micropipette was slowly inserted in the PFC to a depth of 2.1mm from the brain surface. Once the appropriate dorsoventral coordinate was reached, the microinjection was performed slowly (8-10 minutes). After the injection, the glass micropipette remained for 5 min in order to prevent a large spread of the virus. Posteriorly, the same procedure was employed to perform the microinjection in the contralateral hemisphere. Finally, the scalp of the animals was sutured with 3 or 4 stitches, depending on the size of the incision. Animals were then placed back in their homecages and were supplied with tap water containing low doses of metacam (concentration: 2.5mg/ml) for postoperative analgesia during 1 week. After the surgery, the rodents remained for 4 weeks in the animal facility before the experiments to allow a robust infection by the virus.

III.5.3. Intra-PFC drug microinjection

In order to perform the different pharmacological approaches addressed in the present study, a 8mm stainless steel cannula was unilaterally implanted in the PFC during the stereotaxic surgery, according to the aforementioned antero-posterior and medio-lateral coordinates. The cannula was lowered to a depth of 1.1mm from the brain surface in order to prevent any lesion of the PFC. In addition, a second hole was drilled near the cannula and a small screw (length: 1.2 mm, diameter: 2 mm, custom-built from stainless steel, Paul Korth GmbH, Germany) was screwed to the surface of the skull to further fixate the protective helmet and the cannula. Both the screw and the cannula were fixed to the skull by the application of instant adhesive (UHU GmbH & Co. KG, Bühl, Germany) and dental cement (Paladur, Heraeus Kulzer GmbH, Germany). The dental cement covered the whole surface of the exposed skull and built a protective helmet to prevent movement of the cannula. After the surgery, animals had a recovery period of 1 week before the corresponding experiments. To minimize suffering, they received further analgesic treatment for 5 days by diluting Metacam (concentration: 2.5mg/ml) in the drinking water.

III.5.4. Drugs

According to the pharmacological approach employed, animals received intra-PFC microinjections of the following drugs: CRH (0.6mM or 0.1μM; Ferring Arzneimittel), Astressin (1 μM; provided by Prof. JeanE. Rivier, The Salk Institute.) or Rp-cAMPS (22mM; Sigma – Aldrich).

III.6. Analytics

III.6.1. *In Situ* Hybridization

Frozen brains of either stressed or control animals were sectioned in 18μm thick slices containing the PFC or motor and somatosensory cortex at -20°C in a cryostat, subsequently mounted on Super Frost slides, dried and stored at -80°C. *In Situ* hybridization was performed on these slices using a S UTP labeled ribonucleotide probe for the exon 12 of the CRHR1 (Nucleotides 1728-2428 of GenBank accession no. NM_007762; Transcript size: 702 bp) as previously described (Refojo et al., 2011b). The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed following 6 days of exposure. Autoradiographs were digitized, and expression was determined by optical densitometry utilizing the freely available NIH ImageJ software. The mean of two measurements of two different brain slices were calculated for each animal. The data was analyzed blindly, always subtracting the background signal of a nearby structure not expressing CRHR1 from the measurements.

III.6.2. CRHR1 Autoradiography

The CRHR1 autoradiography was performed according to Greetfeld et al., 2009. Glass slides containing PFC tissue sections were thawed and vacuum-dried overnight at 4°C. The next day, they were fixed in 0.1% PFA for 2 min and then incubated in 0.1% BSA for 45 min at room temperature. Two sets of PFC sections were prepared: One for CRHR1 binding and the other to detect non-specific binding. To detect CRHR1, slides were incubated in 0.5mM Antisauvagine (Sigma-Aldrich) for 60 min. Subsequently, they were incubated in 0.2 nM ^{125}I(Tyr^{0})Sauvagine (Perkin Elmer) for 2 hrs and then washed in a buffer containing 50mM TRIZMA- Base, 10mM $MgCl_2$ ($6H_2O$), 2mM EGTA, 0.1mM Bacitracin, 100 KIU/ml Trasylol (Sigma-Aldrich) at 4°C, twice for 5 min. To quantify non-specific binding, a separate set of adjacent sections was incubated in 50 μM Sauvagine

(Sigma-Aldrich), 0.1%-BSA and 0.05 nm $^{125}I(Tyr^{0})$Sauvagine for 2 hrs. Dried slices were placed on a Kodak Biomax MR Film (Eastman Kodak Co., Rochester, NY, USA) for 6 days. CRHR1 binding within the PFC was determined by optical densitometry using the freely available NIH ImageJ software. The mean of two measurements of two different brain slices were calculated for each animal. The data was analyzed blind to treatment (Empty vs Cre), always subtracting the background signal of a nearby structure not expressing CRHR1. Posteriorly, the results from CRHR1 binding were subtracted from the ones obtained in non-specific binding slides.

III.6.3. Western blot analysis

Protein extracts were obtained by lysing cells in lysis buffer containing 62.5 mM Tris, 2% sodium dodecyl sulfate and 10% sucrose, completed with protease inhibitor cocktail (Sigma, P2714) and phosphatase inhibitor (Roche, 04906837001). After sonication and heating for 5 min at 95 °C, samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes by electrotransfer. Membranes were blocked in Tris-buffered saline, supplemented with 0.05% Tween (Sigma, P2287) and 5% non-fat milk for 1 h at room temperature. Incubation with primary antibodies was carried over night at 4°C. Subsequently, membranes were washed and probed with respective fluorophore- or horseradish peroxidase-conjugated secondary antibody for 3-5 h at room temperature. Detectable, immunoreactive bands were visualized either by excitation of respective fluorophore or by using ECL reagent (Millipore, WBKL0500). Detection and quantification of band intensities were performed with ChemiDoc MP (BioRad). For quantification of pCREB and pERK, the intensity was always referred to the signal intensity of total CREB and ERK respectively.

III.6.4. Co-immunoprecipitation (coIP)

Immunoprecipitations of endogenous PKA-C were performed in protein lysates of mouse PFC. The tissues were lysed in coIP buffer containing 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1mM EDTA, 0.5% Igepal complemented with protease inhibitor cocktail (Sigma, P2714), followed by incubation for 20 min at 4 °C while shaking on an overheadshaker. To clear the lysate, samples were centrifuged and protein concentration was determined. Overnight, 500 μg of lysates were incubated with 2 μg of PKA-C antibody at 4 °C. In all, 20 μL of protein G dynabeads (Invitrogen, 100-03D) were blocked with bovine serum albumin and subsequentially added to the lysate-antibody mix, followed by incubation at

4°C for 3 h. The beads were washed three times with ice-cold phosphate-buffered saline (PBS). The protein-antibody complexes were eluted with 60 μL Laemmli loading buffer, while boiling for 5 min at 95 °C. In total, 2-5 μL of immunoprecipitates or 15-20 μg of tissue lysates were separated by SDS-PAGE and electro-transferred onto nitrocellulose membranes. Primary anti-CREB, anti-PKa-C and anti-PKa-RI were used for assessing protein complexes.

III.7. Clarity

In order to visualize the CRHR1- and CRH-containing networks within the PFC, the clarity procedure was employed. This method allows the transformation of intact tissue into a nanoporous hydrogel-hybridized form that is fully assembled but optically transparent. Mice were deeply anesthezised with Narcoren and then intracardially perfused first with 20ml of 0.1M PBS at 4°C and then with 20ml of a hydrogel solution containing 4% Acrylamide, 0.05% Bis, 0.25% VA-044 Initiator, 4% PFA and 0.1M PBS at 4°C. Immediately after, the brain was extracted and incubated in hydrogel solution at 4°C for 3 more days. Then, it was further incubated at 37°C for 3h, until the hydrogel solution had polymerized. After embedding, a 1mm thick section of the PFC was extracted using a brain matrix. Posteriorly, the tissue was submitted to a clearing process in which the sample was washed in a clearing solution containing 200mM Boric Acid and 4% Sodium Dodecyl Sulfate with a 8.5 pH for 2 days at 37°C. After another week of incubation in clearing solution at 37°C the sample was washed 2 times, of 24 hrs each, with PBST (0.1% TritonX in 0.1M PBS). Once concluded this procedure, the tissue was submitted to an immunofluorescence protocol. The tissue was incubated with the primary antibody against GFP in 0.1M PBS/0,3%TritonX for 24 hrs at 37°C followed by a 4 night incubation at 4°C. Later, the sample was washed in 0.1M PBS /0,1%TritonX for 2 days at room temperature. Images were acquired using the LaVision Light Sheet microscope to get an overview of the sample. Posteriorly, the sample was taken to an Olympus Confocal microscope in order to acquire single cell images.

III.8. Antibodies

The following primary antibodies were used: anti-CREB (1:1000, Rabbit, Cell Signaling Technology, #9197), anti-pCREB (1:1000, Rabbit, Cell Signaling Technology, #9198), anti-

pERK (1:1000, Cell Signaling Technology, #9101), anti-ERK (1:1000, Cell Signaling Technology, #9102), anti-PKa Cα (1:1000, rabbit, Cell Signaling Technologies, #4782) and anti-PKa RI (1:1000, rabbit, Cell Signaling technologies, #5675), anti-actin (1:5000, Santa Cruz Biotechnologies, sc-1616), anti GFP (rabbit 1:50, fluorophore labeled Invitrogen #A21311). Secondary antibodies were donkey anti-goat immunoglobulin G, Alexa Flour 488 (1:7000, Life Technologies, A-11055), anti-rabbit immunoglobulin G, horseradish peroxidase-linked (1:10000, Cell Signaling Technologies, #7074) and anti-mouse immunoglobulin G, horseradish peroxidase-linked (1:10000, Cell Signaling Technologies, #7076).

III.9. Statistics

The data presented are shown as means + standard error of the mean, analyzed by the commercially available softwares GraphpadPrism 4 and SigmaStat 3.5. For within group comparisons (old vs recent object exploration; training vs retention session), a two-tailed paired *t* test was employed. To calculate differences between two independent groups (control vs stress; ACSF vs CRH), they were tested for normal distribution and when appropriately analyzed with a two-tailed unpaired *t* test was used. To analyze more complex datasets (2 x 2 design: CRHR1 allele x stress; CRH x Astressin; CRH x Rp-cAMPS), a 2-way ANOVA was performed, followed, when appropriate, by a Bonferroni *post hoc* test. As nominal level of significance $p < 0.05$ was accepted. Values outside the 95% confidence interval (CI) were defined as statistical outliers and excluded from the analyses.

IV. RESULTS

IV.1. Experiment 1: Effects of acute stress in PFC-mediated cognition

IV.1.1. Effects of acute stress in temporal order memory

Several lines of evidence have shown acute stress-induced impairments in different cognitive processes (Jakobsson and Gustavsson, 2014). However, few studies have focused on the PFC. Furthermore, the effects of acute stress on high-order memory processes remain largely unexplored. Particularly, temporal order memory encompasses recognition memory processes based on the relative familiarity of objects, using recency information. In both, humans and rodents, this cognitive process has been shown to depend on the integrity of PFC networks. In order to assess the effects of acute stress in such PFC-mediated task, the temporal order memory test was employed.

Animals were submitted to a single 5-minute episode of social defeat stress, and 5.5 hours later, underwent the sample phase I, sample phase II and test phase with an inter-phase interval of 1 hour (Figure 7A). The results obtained during the initial 2 sample phases show no significant differences on the exploration times between phase I and II for control and stressed animals, suggesting an absence of a preference towards the objects presented on each phase (Figure 7B). Additionally, there is no effect of stress on the exploration of the objects during sample phase I and II, indicating that both groups of animals explored similarly across the different stages of the test (Figure 7B). On the other hand, during the test phase, control animals exhibited a significantly increased exploration of the old object versus the recent object (paired *t* test, $t_{(10)}= 5.402$, $p<0.001$), whereas stressed animals showed no significant differences between the exploration of the old and recent objects, suggesting an impairment in temporal order memory. Furthermore, acute stress induced a significant reduction in the percentage of exploration of the old object (unpaired *t* test, $t_{(21)}= 4.186$, $p<0.001$) compared with control animals (Figure 7C). Accordingly, acute stress evoked a significant decrease in the discrimination ratio between the old and recent objects during the test phase (unpaired *t* test, $t_{(21)}= 4.193$, $p<0.001$), indicating an impairment in the ability to discriminate past events based on their relative recency (Figure 7D).

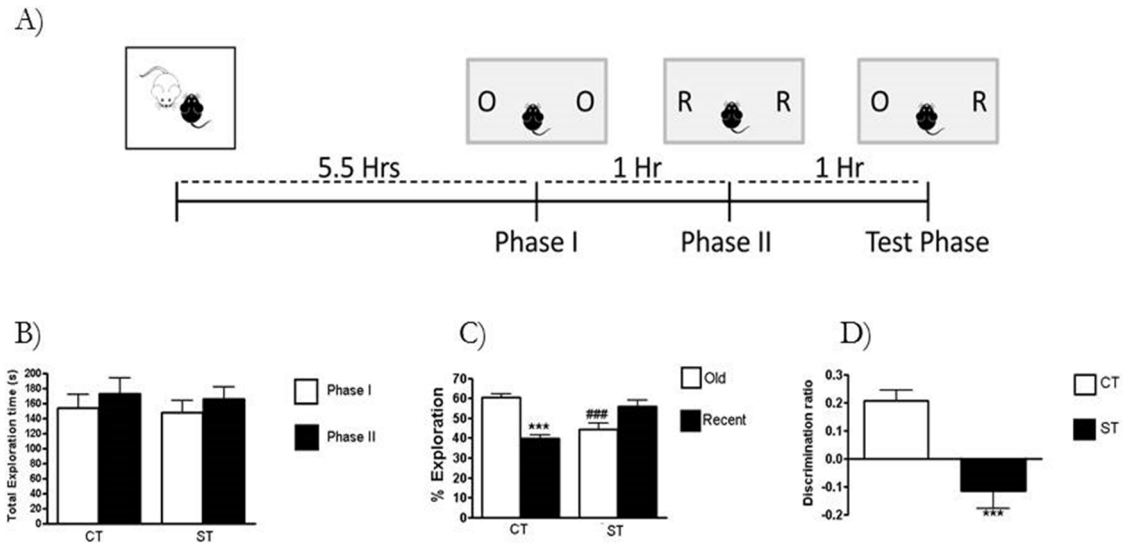

Figure 7 Effects of acute social defeat stress in temporal order memory.

A) Experimental design. O= Old Object; R= Recent Object. B) Exploration time of the objects presented during sample phases I and II of the temporal order memory test. C) Percentage of exploration of the old and recent objects presented during the test phase of the temporal order memory test. *** Significantly different within the same group, $p<0.001$; ### Significantly different from the percentage of exploration of the old object in control animals, $p<0.001$. D) Discrimination ratio between the old and recent objects presented during the test phase of the temporal order memory test. *** Significantly different from the control animals, $p<0.001$. CT= Control; ST= Stressed.

IV.1.2. Effects of acute stress in reversal learning

In order to further delineate the impact of acute stress in executive functions, a separate batch of animals was submitted to the training and retention sessions of the reversal learning test. On the 3rd day, rodents underwent a single episode of social defeat stress, and 6 hours later went through the reversal session of the test (Figure 8**¡Error! No se encuentra el origen de la referencia.**A). The data show a significant decrease in the trials to reach criteria between the training and retention sessions (paired *t* test, $t_{(19)}= 2.923$, $p<0.05$), indicating a robust consolidation of the behavioral response (Figure 8**¡Error! No se encuentra el origen de la referencia.**B). Additionally, stress has no effect in the number of perseverative errors, or mistakes made before making the first correct response, during the reversal session of the test (Figure 8**¡Error! No se encuentra el origen de la referencia.**C). However, acute stress induces a significant increase in the number of trials to reach criteria during the reversal phase (unpaired *t* test, $t_{(18)}= 2.238$, $p<0.05$), indicating

an alteration in the ability to switch a predominant behavioral pattern for an opposite one, according to environmental feedback (Figure 8D).

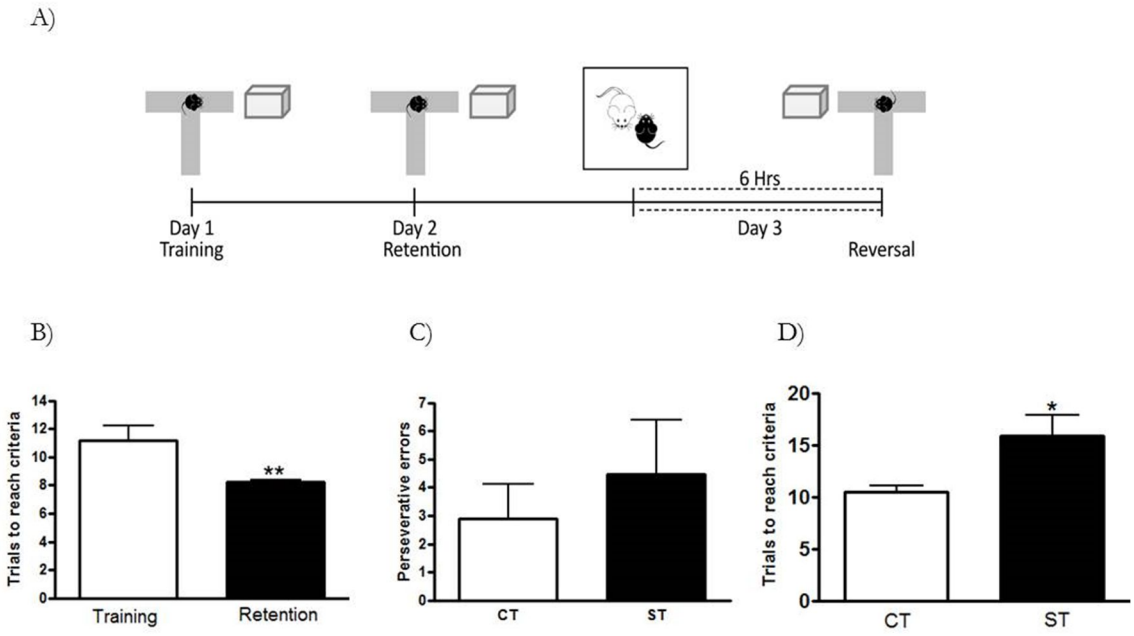

Figure 8 :Effects of acute social defeat stress on reversal learning.

A) Experimental design. B) Trials to reach criteria during the training and retention session. ** Significantly different from the training session, $p<0.01$. C) Number of perseverative errors during the reversal session. D) Trials to reach criteria during the reversal session. CT= Control; ST= Stressed. * Significantly different from the control group, $p<0.05$.

IV.2. Experiment 2: Visualization of the CRH- and CRHR1-containing neural circuits within the PFC networks

The robust presence of CRH-containing neurons in the PVN, together with the strong expression of CRHR1 in the anterior pituitary, have nourished a series of experiments elucidating the role played by the CRH-CRHR1 system in the fast activation of the HPA axis, leading to the initiation of the stress response (Bonfiglio et al., 2013). Furthermore, additional studies focusing on the same neurochemical system have provided evidence of several extra-hypothalamic CRH synthesis sites, including basolateral amygdala (BLA) and cortex. As well, similar approaches have clearly identified CRHR1-expressing neurons outside the HPA axis, along the entire brain, encompassing cortex, hippocampus, olfactory bulb and several other relevant regions (Reul and Holsboer, 2002). Such findings suggest that besides playing a key role in HPA axis stimulation, the CRH-CRHR1 system can

actually constitute an essential contributor to several stress-induced cognitive alterations, including PFC-mediated cognitive functions. However, in order to further address this hypothesis, it is essential to clearly identify the presence of CRH- and CRHR1-expressing neurons within the PFC networks. To achieve this goal, PFC sections of animals belonging to the CRFcre x Ai9 x R1GFP mouseline, expressing the red fluorescent protein tdTomato in CRH neurons and GFP in CRHR1-expressing neurons, were submitted to the clarity protocol (see III.7).

The results obtained show that both, CRH and CRHR1, are widely expressed across different prosencephalic cortical regions and PFC subregions, including AC, PL and IL. In this regard, the images acquired exhibit a robust expression of CRH and CRHR1 along the midline portion of these PFC-related structures. Whereas CRHR1 expression seems to be densely clustered, CRH-expressing cell bodies present a wider distribution across the medio-lateral axis, scattering towards more medial and lateral aspects of the aforementioned regions. In addition, the present findings show that despite the regional convergence of CRH and CRHR1, there is a well-defined, intra-regional spatial segregation between both cell-types. As a matter of fact, there is no overlapping between CRH and CRHR1-expressing cells, supporting the notion of CRH-CRHR1 microcircuits within PFC networks, which could be responsible for the acute stress-induced, CRHR1-mediated, executive dysfunction (Figure 9).

IV.3. Experiment 3: Effects of acute stress on CRHR1 mRNA expression in the PFC

Acute stress-induced cognitive alterations have been shown to depend on the activity of several stress-related neurochemical systems. Thus, previous reports have established the role of the CRH system, particularly through CRHR1 signaling, in stress-induced morphological and cognitive alterations in the hippocampus (Wang et al., 2013). To establish the potential relevance of CRHR1 in the executive dysfunction observed after acute stress, animals were submitted to acute social defeat stress or control conditions and sacrificed 8 hours later (Figure 10A). Brain sections of these animals, containing either the PFC or motor and somatosensory cortex, were then submitted to an *In Situ* Hybridization protocol to detect changes in CRHR1 mRNA levels. The results obtained show significant increases in CRHR1 mRNA in the cingulate cortex (unpaired *t* test, $t_{(22)}= 2.978$, $p<0.01$),

PL (unpaired *t* test, $t_{(22)}$= 2.592, p<0.05) and IL (unpaired *t* test, $t_{(22)}$= 3.009, p<0.01) regions (Figure 10B, C and D). Moreover, no effects on CRHR1 mRNA expression were detected in the motor and somatosensory cortex (Figure 10E, F and G), suggesting that acute stress induces a region-specific CRHR1 upregulation in the PFC, and pointing to its potential involvement in the acute stress-evoked executive dysfunction.

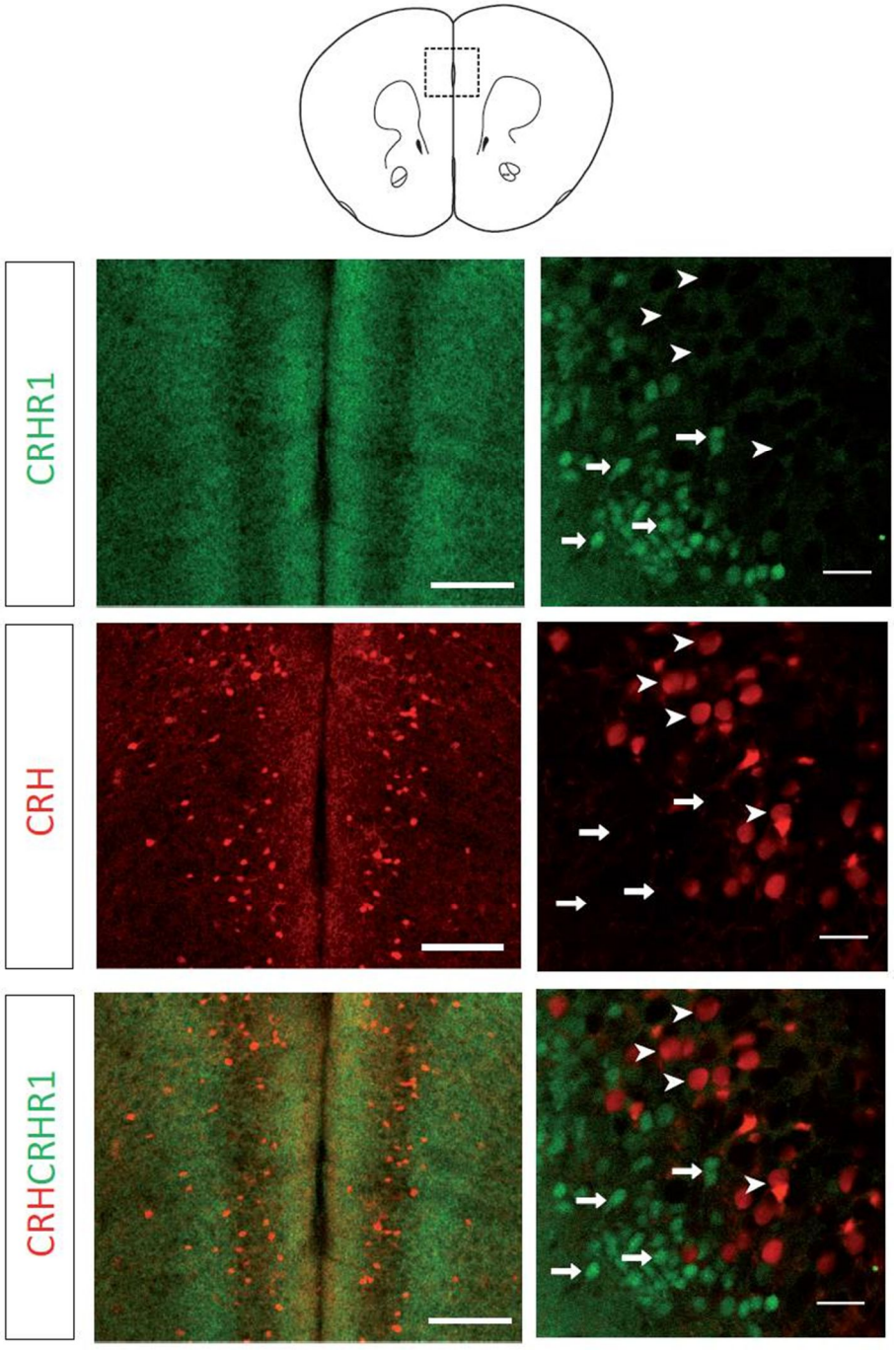

Figure 9 Visualization of the CRH- and CRHR1-containing neural circuits within the PFC networks.

Upper part: Schematic diagram of a coronal section depicting the region of interest (mPFC) in a dashed square. Representative confocal images taken from the mPFC showing CRHR1- and CRH-immunoreactive neurons. Arrows represent CRHR1-expressing neurons whereas arrowheads show CRH-containing cell bodies. Scalebars: Left column 200μm; Right column 30μm.

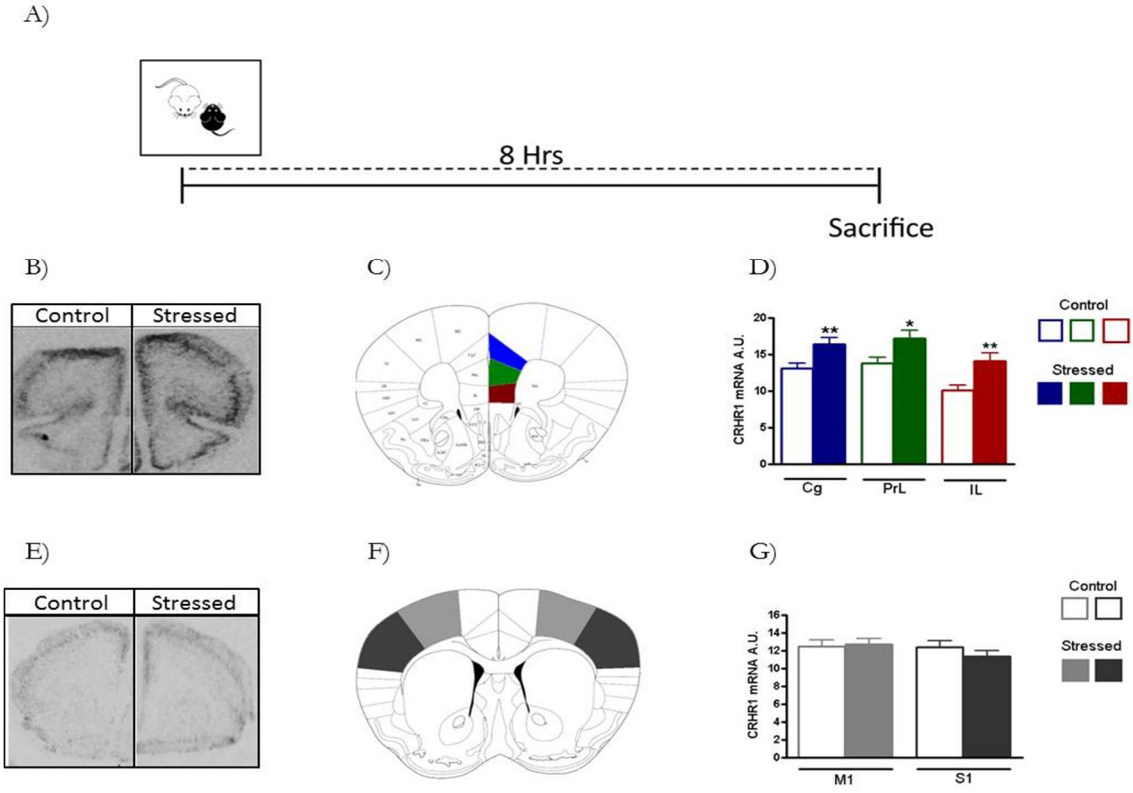

Figure 10 Effects of acute social defeat stress on CRHR1 mRNA expression in the PFC. A) Experimental design. B) CRHR1 *In Situ* Hybridization sample pictures of coronal sections belonging to stressed and control animals, containing the PFC. C) Schematic design of a coronal section depicting different mPFC subregions: Cingulate (Blue), Prelimbic (Green) and Infralimbic (Red). D) Quantification of the CRHR1 mRNA levels after acute social defeat in Cingulate (Cg), Prelimbic (PrL) and Infralimbic (IL) areas. * Significantly different from the control group, $p<0.05$. ** Significantly different from the control group, $p<0.01$. E) CRHR1 In Situ Hybridization sample pictures of coronal sections belonging to stressed and control animals, containing the Somatosensory and Motor regions. F) Schematic design of a coronal section depicting the Somatosensory (Dark gray) and Motor (Light gray) areas. G) Quantification of the CRHR1 mRNA levels after acute social defeat in the Somatosentory (S1) and Motor (M1) regions.

IV.4. Experiment 4: Effects of intra-PFC CRHR1 deletion on the acute stress-induced executive dysfunction

IV.4.1. Effects of intra-PFC CRHR1 deletion on the impact of acute stress in temporal order memory

To further establish the role played by the CRHR1 in the acute stress-induced executive dysfunction, we used a genetic approach employing an adeno-associated virus (AAV) gene transfer strategy. Summarizing, CRHR1$^{loxP/loxP}$ mice (Figure 11A) received intra-PFC microinjections of AAV containing either Cre (referred to as PFC CRHR1-CKO hereafter) or empty (referred to as WT hereafter) expression cassettes. One month after surgery, animals were submitted to a single episode of acute social defeat stress or control conditions and 5.5 hours later underwent the temporal order memory test (Figure 11B). Neither acute stress nor the PFC-specific CRHR1 knockout altered the exploratory activity of the animals during sample phase I or II. In addition, there are no significant differences in object exploration between sample phase I and II for each group of animals, indicating an equivalent exploratory activity across all groups (Figure 11C).

Concerning temporal order memory, the statistical analysis showed a significantly increased percentage of exploration of the old object vs the recent object for control WT (Paired *t* test, $t_{(7)}$= 3.451, p<0.05), control PFC CRHR1-CKO (Paired *t* test, $t_{(9)}$= 3.746, p<0.01) and stressed PFC CRHR1-CKO rodents (Paired *t* test, $t_{(9)}$= 7.572, p<0.0001). On the other hand, stressed WT mice exhibited an opposite pattern, exploring more the recent object (Paired *t* test, $t_{(8)}$= 2.339, p<0.05). Additionally, the 2-way ANOVA revealed a significant effect of stress ($F_{3,33}$=9.235, p<0.01), CRHR1 ($F_{3,33}$= 14.464, p<0.001) and stress x CRHR1 interaction ($F_{3,33}$= 12.891, p<0.01). Bonferroni *post-hoc* test showed a significant decrease in the percentage of exploration of the old object for the stressed WT compared with the control WT (p<0.001). As well, *post hoc* testing revealed that stressed PFC CRHR1-CKO animals explored significantly more the old object compared to stressed WT mice (p<0.001) (Figure 11D). Furthermore, the 2-way ANOVA showed an effect of stress ($F_{3,33}$=9.223, p<0.01), CRHR1 ($F_{1,33}$= 14.457, p<0.001) and stress x CRHR1 interaction ($F_{3,33}$= 12.906, p<0.01) in the discrimination ratio between the old and recent object during the test phase of the temporal order memory test. Subsequent *post hoc* testing showed a significant decrease in the stressed WT compared with the control WT (p<0.001), accompanied by a significant increase in the stressed PFC CRHR1-CKO, compared with the stressed WT group (p<0.001) (Figure 11E).

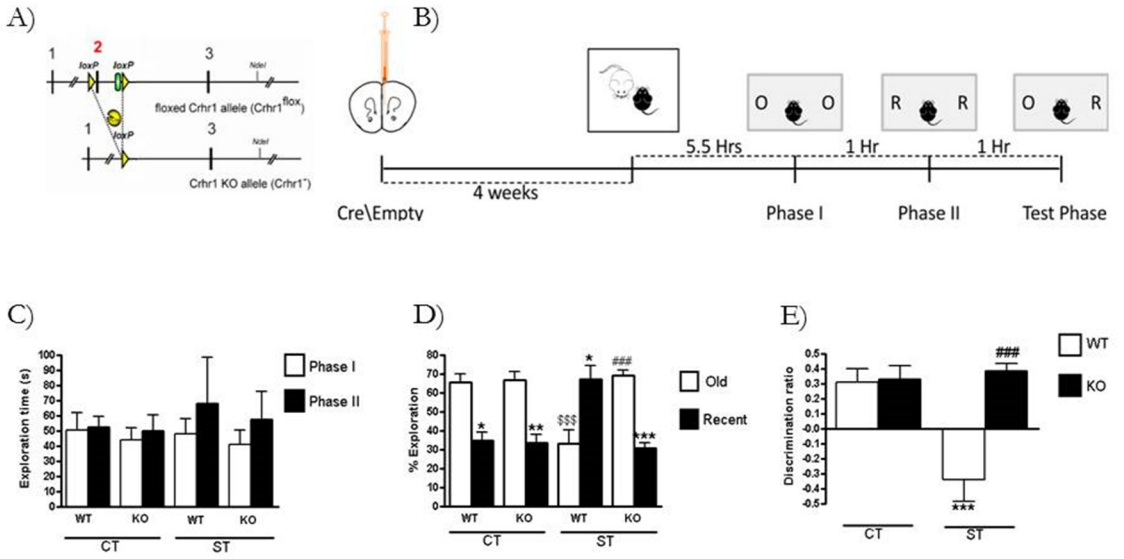

Figure 11 Effects of intra-PFC CRHR1 deletion on the impact of acute stress in temporal order memory.

A) Schematic diagram of the targeted manipulation of the CRHR1 locus. Restriction maps of the CRHR1 allele with and without endogenous flox signal peptide following Cre-mediated excision of the loxP-flanked exon 2. B) Experimental design. O= Old Object; R= Recent Object. C) Exploration times of the objects presented during the sample phases I and II of the temporal order memory test. D) Percentage of exploration of the old and recent objects presented during the test phase of the temporal order memory test. * Significantly different within the same group, $p<0.05$. ** Significantly different within the same group, $p<0.01$. *** Significantly different within the same group, $p<0.001$. $$$ Significantly different from the percentage of exploration of the old object in the CT-WT group, $p<0.001$. ### Significantly different from the percentage of exploration of the old object in the ST-WT group, $p<0.001$. E) Discrimination ratio between the old and recent objects presented during the test phase of the temporal order memory test. *** Significantly different from the CT-WT group, $p<0.001$. ### Significantly different from the ST-WT group, $p<0.001$. CT= Control; ST= Stressed; WT= Wildtype CRHR1 allele; KO= Knockout CRHR1 allele.

IV.4.2. Effects of intra-PFC CRHR1 deletion on the impact of acute stress in reversal learning

In addition, the same genetic strategy was employed to assess the role played by the CRHR1 in the acute stress-induced deficits in reversal learning. Thus, PFC CRHR1-CKO and WT animals underwent the training and retention sessions of the reversal learning test. On the 3rd day, they were submitted either to an acute social defeat stress episode or to control conditions, and 6 hours later, were tested in the reversal session (Figure 13A). Both WT (paired *t* test, $t_{(13)}= 3.478$, $p<0.01$) and PFC CRHR1-CKO (paired *t* test, $t_{(18)}=2.970$, $p<0.01$) showed a significant reduction in the number of trials to reach criteria between the

training and retention sessions (Figure 13B). Furthermore, the 2-way ANOVA revealed a significant effect of stress ($F_{(3,29)}$=19.421, p<0.001), CRHR1 ($F_{(3,29)}$=12.714, p<0.01) and stress x CRHR1 interaction ($F_{(3,29)}$=10.792, p<0.01) in the number of perseverative errors made during the reversal session. Subsequent *post hoc* testing showed a significant increase in the stressed WT compared with the control WT rodents (p<0.001), accompanied by a significant decrease in the stressed PFC CRHR1-CKO compared to stressed WT animals (p<0.001) (Figure 13C). Additionally, the 2-way ANOVA revealed a significant effect of stress ($F_{(3,29)}$=9.395, p<0.01), CRHR1 ($F_{(3,29)}$=7.440, p<0.05) and stress x CRHR1 interaction ($F_{(3,29)}$=17.291, p<0.001) in the trials to reach criteria during the reversal session. Subsequent *post hoc* testing showed a significant increase in the stressed WT compared with the control WT mice (p<0.001) accompanied by a significant decrease in the stressed PFC CRHR1-CKO compared with the stressed WT group (p<0.001) (Figure 13D). Posteriorly, PFC coronal sections of all animals were submitted to a CRHR1 autoradiography to detect the effectiveness of the CRHR1-CKO (Figure 13E). The statistical analysis revealed a significant decrease in CRHR1 binding in the PFC of rodents that received AAV-Cre microinjections (unpaired *t* test, $t_{(34)}$=4.620, p<0.0001)(Figure 13 F).

IV.5. Experiment 5: Effects of intra-PFC CRH microinjections in PFC-mediated cognition

IV.5.1. Effects of intra-PFC CRH microinjections in temporal order memory

According to the present findings, the CRHR1 plays a crucial role in the PFC-dysfunction caused by acute stress. Thus, its activation by its main ligand, CRH, must also constitute a key component of the effects initiated by stress. However, little is known about the activity of CRH, and its effects upon cognition, within the PFC networks. In order to further contribute in this direction, we performed a pharmacological approach to establish the effects of intra-PFC CRH on PFC-mediated cognitive processes. Accordingly, C57Bl6 mice were submitted to stereaotaxic surgery and a guide cannula was implanted in the right PFC, encompassing PL and IL regions. One week after surgery, animals received an intra-PFC microinjection of either ACSF or CRH. 5.5 hours after microinjection, the rodents were submitted to the temporal order memory test (Figure 13A). The results obtained during the 2 sample phases show no significant differences on the exploration times between phase I and II for animals with intra-PFC ACSF and CRH microinjections, suggesting an absence

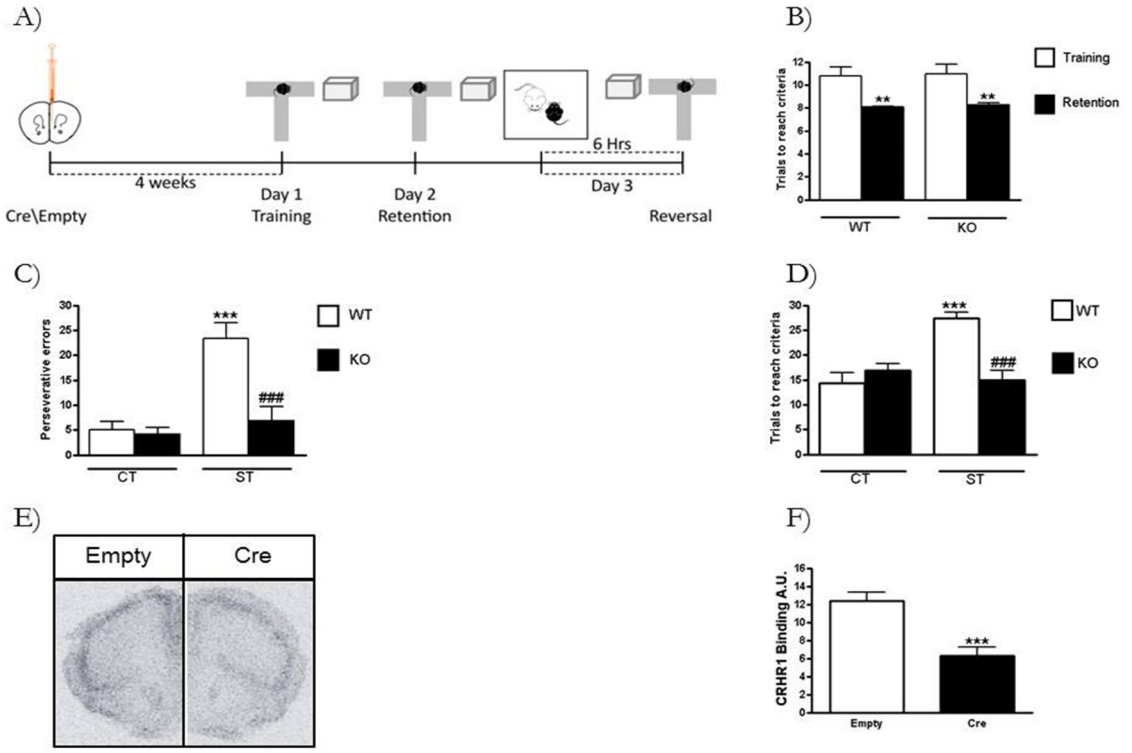

Figure 12 Effects of intra-PFC CRHR1 deletion on the impact of acute stress in reversal learning.

A) Experimental design. B) Trials to reach criteria during the training and retention session. ** Significantly different from the training session within the same group, $p<0.01$. C) Number of perseverative errors during the reversal session. D) Trials to reach criteria during the reversal session. *** Significantly different from the CT-WT group, $p<0.001$. ### Significantly different from the ST-WT group, $p<0.001$. WT= Wildtype CRHR1 allele; KO= knockout CRHR1 allele; CT= Control; ST= Stressed. E) CRHR1 autoradiography sample pictures of coronal sections belonging to CRHR1loxP/loxP animals that received intra-PFC AAV microinjections containing either Empty or Cre expression cassettes. F) Quantification of the intra-PFC CRHR1 binding detected through autoradiography. *** Significantly different from animals with intra-PFC empty-AAV microinjections, $p<0.0001$.

of a preference towards the objects presented on each phase (Figure 13B). Additionally, there is no effect of CRH on the exploration of the objects during sample phase I and II, indicating that both groups of rodents explored similarly across the different stages of the test (Figure 13B). On the other hand, during the test phase, mice with intra-PFC ACSF microinfusions exhibited a significantly decreased exploration of the old object versus the recent object (paired *t* test, $t_{(9)}=2.792$, $p<0.05$), whereas CRH-pretreated animals showed an opposite pattern, exhibiting a significant increase in the exploration of the recent versus the old object (paired t test, $t_{(9)}=3.248$, $p<0.05$), suggesting an impairment in temporal order memory. Furthermore, intra-PFC CRH administration induced a significant reduction in

the percentage of exploration of the old object (unpaired *t* test, $t_{(18)}$=4.177, p<0.001) compared with animals that received intra-PFC ACSF microinjections (Figure 13C). Accordingly, intra-PFC CRH microinfusions evoked a significant decrease in the discrimination ratio between the old and recent objects during the test phase (unpaired *t* test, $t_{(18)}$= 4.910, p<0.001), indicating alterations in the ability to discriminate past events based on their relative recency (Figure 13D).

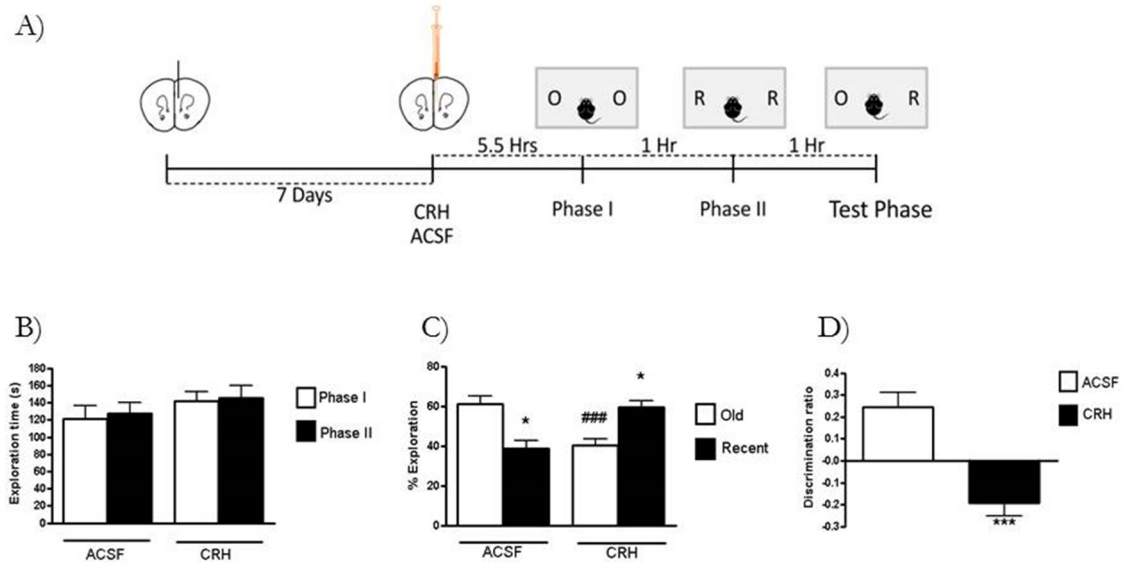

Figure 13: Effects of intra-PFC CRH microinjections in temporal order memory.

A) Experimental design. O= Old Object; R= Recent Object. B) Exploration time of the objects presented during sample phases I and II of the temporal order memory test. C) Percentage of exploration of the old and recent objects presented during the test phase of the temporal order memory test. * Significantly different from the percentage of exploration of the old object within the same group, p<0.05; ### Significantly different from the percentage of exploration of the old object in animals with intra-PFC ACSF, p<0.001. D) Discrimination ratio between the old and recent objects presented during the test phase of the temporal order memory test. *** Significantly different from rodents with intra-PFC ACSF microinfusions, p<0.001.

IV.5.2. Effects of intra-PFC CRH microinjections in reversal learning

Additionally, a separate batch of animals underwent stereotaxic surgery and a guide cannula was implanted in the PFC. One week later, they were submitted to the training and retention sessions of the reversal learning test. The next day, they received intra-PFC microinjections of either ACSF or CRH, and 6 hours later underwent the reversal session of the test (Figure 14A). The data show a significant decrease in the trials to reach criteria between the training and retention sessions (paired *t* test, $t_{(20)}$= 2.490, p<0.05), indicating a

robust consolidation of the behavioral response (Figure 14B). Furthermore, intra-PFC CRH microinfusions had no significant effect in the number of perseverative errors or mistakes made before making the first correct response, during the reversal session of the test (Figure 14C). However, CRH pretreatment induced a significant increase in the number of trials to reach criteria during the reversal phase (unpaired *t* test, $t_{(19)}$=2.114, p<0.05), indicating an alteration in the ability to switch a predominant behavioral pattern for an opposite one, according to environmental feedback (Figure 14D).

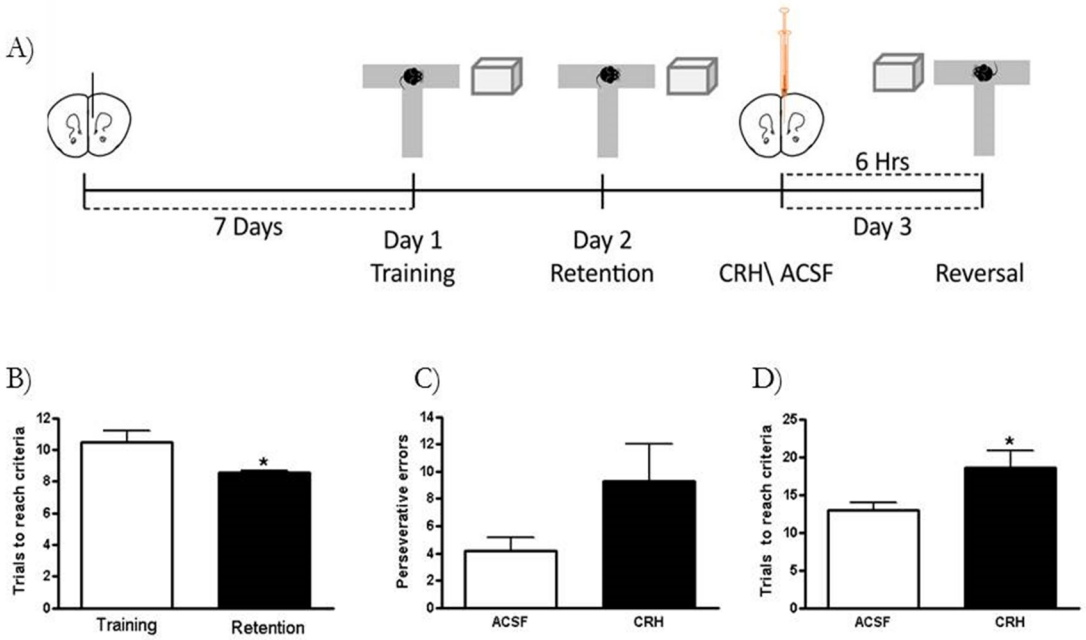

Figure 14: Effects of intra-PFC CRH microinjections on reversal learning.

A) Experimental design. B) Trials to reach criteria during the training and retention session. * Significantly different from the training session, p<0.05. C) Number of perseverative errors during the reversal session. D) Trials to reach criteria during the reversal session. * Significantly different from the ACSF group, p<0.05.

IV.6. Experiment 6: Effects of CRHR1 blockade on the intra-PFC CRH-induced executive dysfunction

IV.6.1. Effects of CRHR1 blockade on the intra-PFC CRH-induced deficits in temporal order memory

In order to further delineate the role of the CRHR1 in the CRH-induced impairments in PFC-related cognitive processes, C57Bl6 rodents were submitted to stereotaxic surgery and a guide cannula was implanted in the PFC. One week after surgery, animals received intra-

PFC microinjections of ACSF, the CRHR antagonist Astressin, CRH or Astressin + CRH. After 5.5 hours, they underwent the temporal order memory test (Figure 15A). None of the microinjected drugs altered the exploratory activity of the mice during sample phase I or II. In addition, there are no significant differences in object exploration between sample phase I and II for each group of animals, indicating an equivalent exploratory activity across all groups (Figure 15B). Concerning temporal order memory, the statistical analysis showed a significantly increased percentage of exploration of the old object vs the recent object for rodents that received Astressin (Paired *t* test, $t_{(7)}$= 3.073, p<0.05) and Astressin + CRH (Paired *t* test, $t_{(7)}$= 6.774, p<0.001). On the other hand, CRH-pretreated mice exhibited an opposite pattern, exploring more the recent object (Paired *t* test, $t_{(9)}$=5.949, p<0.001). Additionally, the 2-way ANOVA revealed a significant effect of Astressin ($F_{3,32}$=13.415, p<0.001), and Astressin x CRH interaction ($F_{3,32}$=19.507, p<0.001). Bonferroni *post-hoc* test showed a significant decrease in the percentage of exploration of the old object for the CRH-pretreated mice compared with the ACSF group (p<0.001). As well, *post hoc* testing revealed that animals that received intra-PFC Astressin + CRH explored significantly more the old object compared to the CRH group (p<0.001) (Figure 15C). Furthermore, the 2-way ANOVA showed an effect of Astressin ($F_{3,32}$=13.529, p<0.001), and Astressin X CRH interaction ($F_{3,32}$=19.450, p<0.001) in the discrimination ratio between the old and recent object during the test phase of the temporal order memory test. Subsequent *post hoc* testing showed a significant decrease in the CRH pretreated rodents compared with the ACSF (p<0.001), accompanied by a significant increase in Astressin + CRH mice compared with animals that received intra-PFC CRH microinjections (p<0.001) (Figure 15D).

IV.5.2 Effects of CRHR1 blockade on the intra-PFC CRH-induced deficits in reversal learning

In addition, the same pharmacological strategy was employed to assess the role played by the CRHR1 in the intra-PFC CRH-induced deficits in reversal learning. Thus, animals with a cannula implanted in the PFC underwent the training and retention sessions of the reversal learning test. On the 3rd day, they received intra-PFC microinjections of ACSF, Astressin, CRH or Astressin + CRH, and 6 hours later were tested in the reversal session (Figure 16A). All animals exhibited a significant reduction in the number of trials to reach criteria between the training and retention sessions (Paired *t* test, $t_{(34)}$=5.681, p<0.0001) (Figure 16B). Furthermore, the 2-way ANOVA revealed a significant CRH x Astressin interaction ($F_{(3,29)}$ = 4.218, p<0.05) in the number of perseverative errors made during the

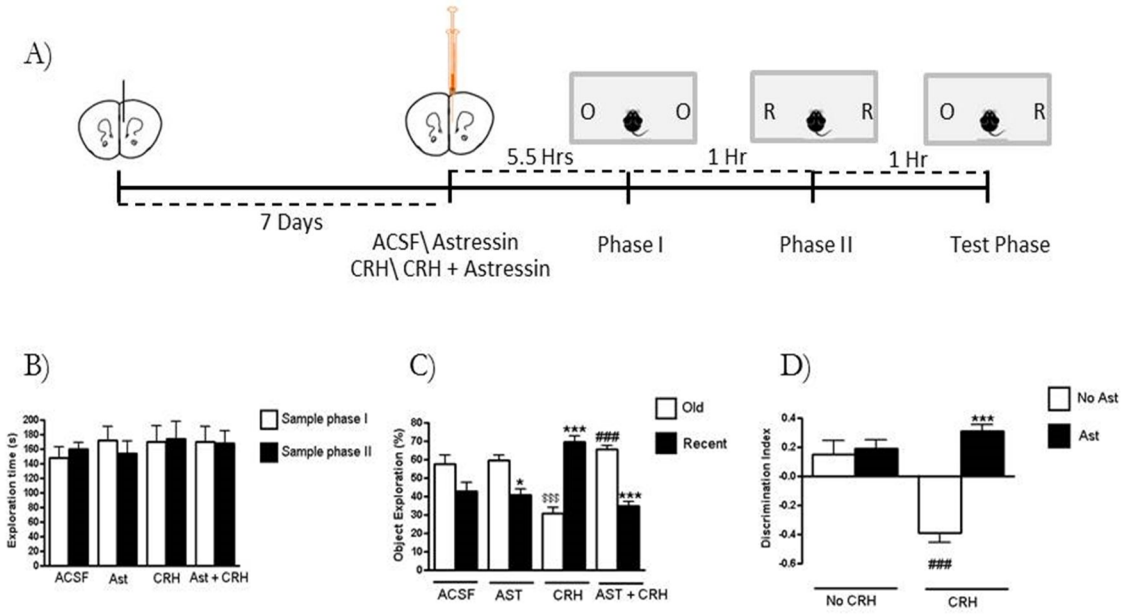

Figure 15: Effects of CRHR1 blockade on the intra-PFC CRH-induced deficits in temporal order memory.

A) Experimental design. O= Old Object; R= Recent Object. B) Exploration times of the objects presented during the sample phases I and II of the temporal order memory test. C) Percentage of exploration of the old and recent objects presented during the test phase of the temporal order memory test. * Significantly different within the same group, $p<0.05$. *** Significantly different within the same group, $p<0.001$. $$$ Significantly different from the percentage of exploration of the old object in the ACSF group, $p<0.001$. ### Significantly different from the percentage of exploration of the old object in the CRH group, $p<0.001$. AST= Astressin. D) Discrimination ratio between the old and recent objects presented during the test phase of the temporal order memory test. *** Significantly different from the CRH – No Ast group, $p<0.001$. ### Significantly different from the No CRH-No Ast group, $p<0.001$. Ast= Astressin.

reversal session. Subsequent *post hoc* testing showed a significant increase in the animals that received CRH compared with the ACSF group ($p<0.05$), accompanied by a significant decrease in the Ast + CRH compared to the CRH animals ($p<0.05$) (Figure 16C). Additionally, the 2-way ANOVA revealed a significant effect of Astressin ($F_{(3,32)}=15.994$, $p<0.001$), CRH ($F_{(3,32)}=8.555$, $p<0.01$) and Astressin x CRH interaction ($F_{(3,32)}=17.245$, $p<0.001$) in the trials to reach criteria during the reversal session. Subsequent *post hoc* testing showed a significant increase in the CRH-pretreated mice compared with the ACSF group ($p<0.001$), accompanied by a significant decrease in rodents with Astressin + CRH microinjections compared with the CRH group ($p<0.001$) (Figure 16D).

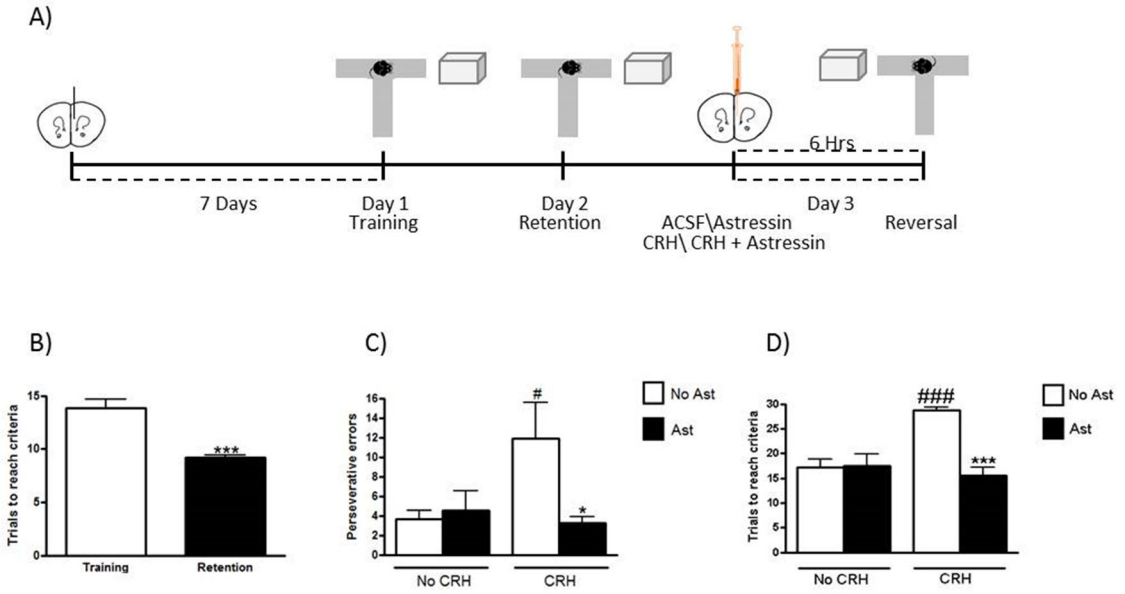

Figure 16: Effects of CRHR1 blockade on the intra-PFC CRH-induced deficits in reversal learning.

A) Experimental design. B) Trials to reach criteria during the training and retention session. *** Significantly different from the training session, $p<0.0001$. C) Number of perseverative errors during the reversal session. D) Trials to reach criteria during the reversal session. ### Significantly different from the No CRH-No Ast group, $p<0.001$. *** Significantly different from CRH-No Ast, $p<0.001$. Ast= Astressin.

IV.7. Experiment 7: Effects of acute stress in the activity of protein kinase A

Several lines of evidence have indicated diverse molecular cascades activated by stress within the PFC, particularly mediated through PKA and PKC. The PKA constitutes a tetramer assemble composed by two regulatory subunits (PKA-R) and two catalytic subunits (PKA-C). Upon activation by cAMP, the complex dissociates and the PKA-Cs are released and translocated into the nucleus. One of PKA-C's main functions is protein synthesis, which is accomplished through the phosphorylation of the transcription factor CREB within the nucleus. To determine the signaling pathway stimulated by acute stress within the PFC networks, C57Bl6 mice were submitted to control conditions or a single episode of acute social defeat stress for 5 minutes. 15 minutes later, animals were sacrificed and their PFC was subsequently prepared and processed in order to analyze protein-protein interactions, within the PKA pathway, through CoIP (Figure 17A). The results obtained show that acute stress induces a significant decrease in the PKA-R to PK-C binding

(unpaired *t* test, $t_{(14)}$=2.246, p<0.05) (Figure 17B and C). In addition, stressed rodents exhibit a significant increase in the interaction between PKA-C and CREB (unpaired *t* test, $t_{(14)}$=2.427, p<0.05), suggesting the activation of the PKA cascade (Figure 17D). Moreover, to further confirm the effects of acute stress on PKA activity, PFC tissue of control and stressed mice was submitted to a western blot protocol to detect changes in CREB phosphorylation after stress. The data revealed a significant increase in the levels of pCREB in stressed animals (unpaired *t* test, $t_{(14)}$=2.368, p<0.05)(**¡Error! No se encuentra el origen de la referencia.**E and F). Interestingly, there is a lack of significant effects on the levels of pERK, another signaling pathway usually activated by stress and related to PKA (Figure 17G and H). This indicates a specific stimulation of the PKA-CREB pathway in the PFC following a single episode of stress.

IV.8. Experiment 8: Effects of intra-PFC CRH microinjections in the activity of protein kinase A

To further determine the signaling pathway stimulated by acute stress, via CRHR1, within the PFC networks, a group of C57Bl6 mice were submitted to stereotaxic surgery and a guide cannula was implanted in the PFC. One week after surgery, they received intra-PFC microinjections of either ACSF or CRH and were sacrificed 15 minutes later. Their PFC was subsequently prepared and processed in order to analyze protein-protein interactions, within the PKA pathway, through CoIP (Figure 18A). The results obtained show that intra-PFC CRH induces a significant decrease in the PKA-R to PK-C binding (unpaired *t* test, $t_{(11)}$=2.344, p<0.05) (Figure 18B and C). In addition, CRH-pretreated rodents exhibit a significant increase in the interaction between PKA-C and CREB (unpaired *t* test, $t_{(11)}$=2.266, p<0.05), suggesting the activation of the PKA cascade (Figure 18D). Moreover, to further confirm the effects of intra-PFC CRH on PKA activity, PFC tissue of ACSF and CRH pretreated mice was submitted to a western blot protocol to detect changes in CREB phosphorylation after CRH. The data revealed a significant increase in the levels of pCREB after CRH microinjections (unpaired *t* test, $t_{(11)}$=2.209, p<0.05)(Figure 18E and F). Similarly to the results observed after acute stress, there is a lack of significant effects on the levels of pERK (Figure 18G and H). This indicates a specific stimulation of the PKA-CREB pathway in the PFC following a single CRH microinjection.

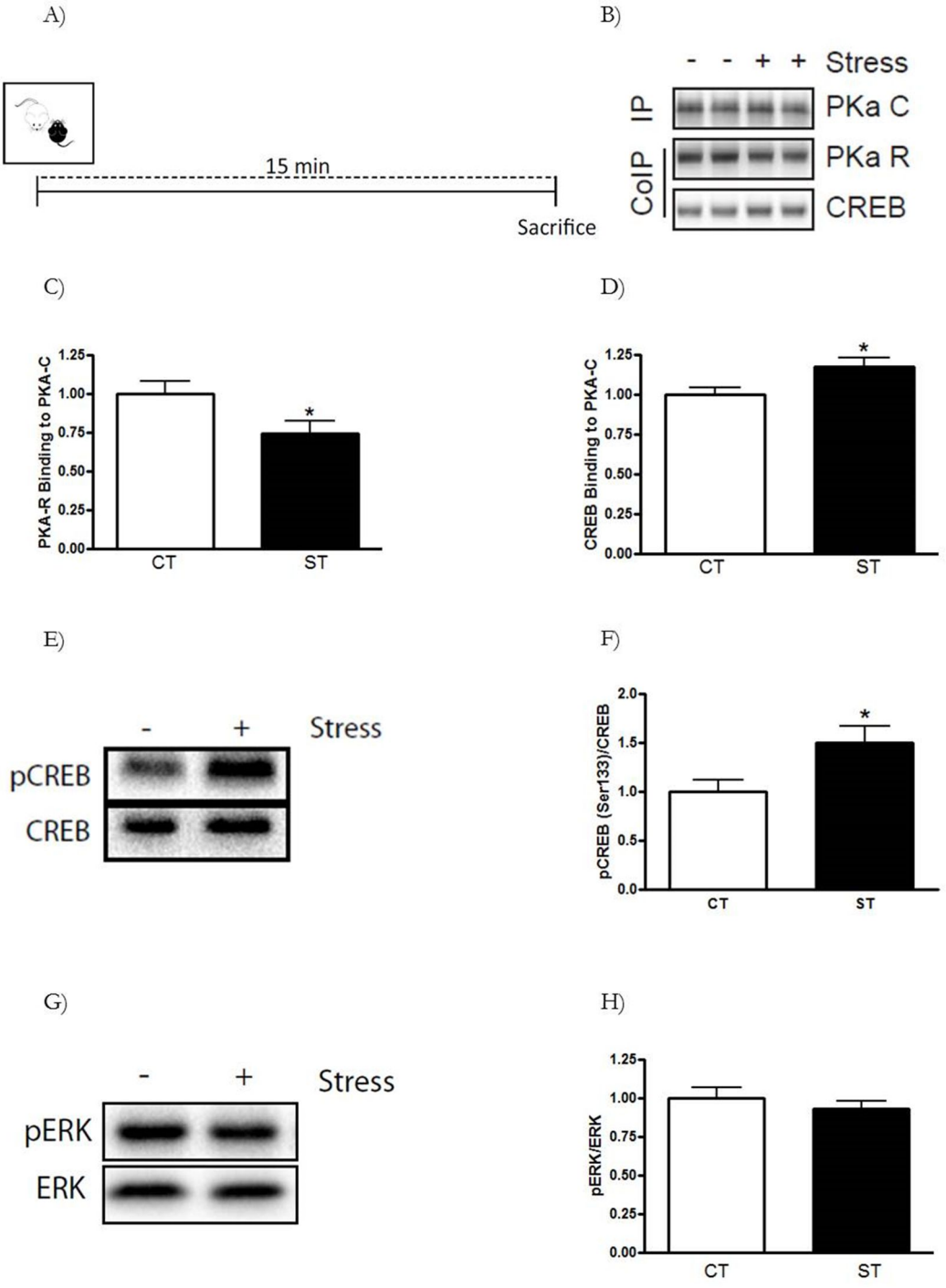

Figure 17: Effects of acute stress in the activity of protein kinase A.

A) Experimental design. B) Sample pictures depicting the results of the co-immunoprecipitation performed to detect PKA-C to PKA-R and PKA-C to CREB binding after acute stress. C) PKA-R to PKA-C binding D) PKA-C to CREB binding. E) Sample pictures depicting the results of the western blot performed to detect changes in pCREB levels after acute stress. F) Levels of pCREB after acute stress. * Significantly different from control animals, $p<0.05$. G) Sample pictures depicting the results of the western blot performed to detect changes in pERK levels after acute stress. H) Levels of pERK after acute stress. CT= Control; ST= Stressed.

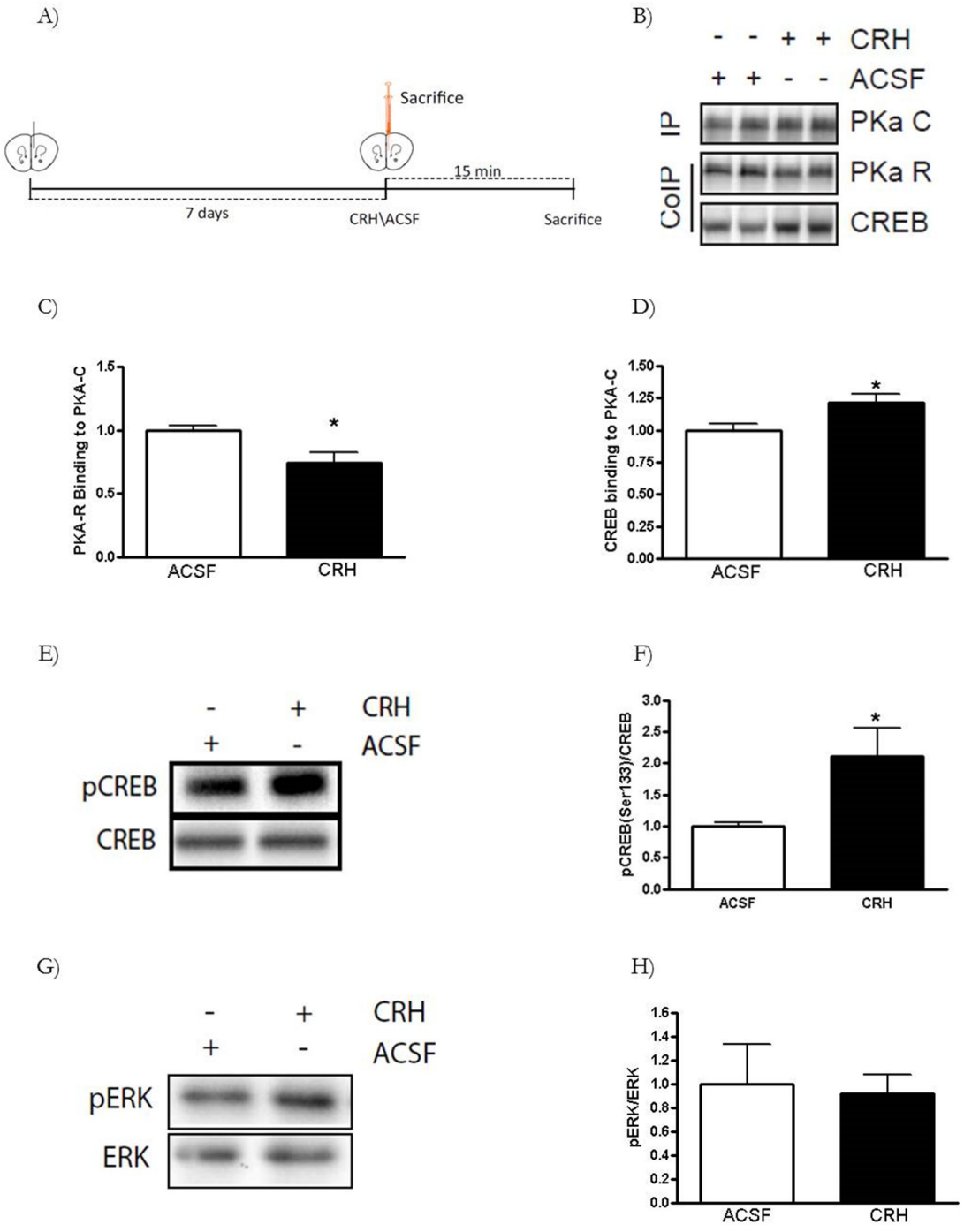

Figure 18: Effects of intra-PFC CRH microinjections in the activity of protein kinase A.

A) Experimental design. B) Sample pictures depicting the results of the co-immunoprecipitation performed to detect PKA-C to PKA-R and PKA-C to CREB binding after intra-PFC CRH administration. C) PKA-R to PKA-C binding D) PKA-C to CREB binding. E) Sample pictures depicting the results of the western blot performed to detect changes in pCREB levels after intra-PFC CRH. F) Levels of pCREB after intra-PFC CRH. * Significantly different from control animals, $p<0.05$. G) Sample pictures depicting the results of the western blot performed to detect changes in pERK levels after intra-PFC CRH. H) Levels of pERK after intra-PFC CRH.

IV.9. Experiment 9: Effects of PKA blockade on the intra-PFC CRH-induced executive dysfunction.

IV.9.1. Effects of PKA blockade on the intra-PFC CRH-induced deficits in temporal order memory

Few reports in the literature have demonstrated the involvement of PKA in stress-induced PFC disruption. Moreover, such studies still lack a causal link between stress-related neurochemical systems, PKA activity and executive dysfunction. Aiming to fill such gap, I submitted C57Bl6 mice to stereotaxic surgery and implanted a guide cannula in the PFC. One week after surgery, animals received intra-PFC infusions of ACSF, the PKA inhibitor Rp-cAMPS, CRH or CRH + Rp-cAMPS. After 5.5 hours, they underwent the sample phase I and II of the temporal order memory test (Figure 19A). None of the microinjected drugs altered the exploratory activity of the mice during sample phase I or II. In addition, there are no significant differences in object exploration between sample phase I and II for each group of animals, indicating an equivalent exploratory activity across all groups (Figure 19B). Regarding temporal order memory, the statistical analysis showed a significantly increased percentage of exploration of the old object vs the recent object for rodents that received ACSF (Paired *t* test, $t_{(11)}$=2.483, p<0.05), Rp-cAMPS (Paired *t* test, $t_{(11)}$=5.511, p<0.001) and a combination of CRH + Rp-cAMPS (Paired *t* test, $t_{(11)}$=6.535, p<0.0001). On the other hand, CRH-pretreated mice exhibited an opposite pattern, exploring more the recent object (Paired *t* test, $t_{(10)}$=8.987, p<0.0001). Additionally, the 2-way ANOVA revealed a significant effect of Rp-cAMPS ($F_{3,43}$=39.366, p<0.001), CRH ($F_{3,43}$=13.286, p<0.001) and RpcAMPS x CRH interaction ($F_{3,43}$=28.128, p<0.001). Bonferroni *post-hoc* test showed a significant decrease in the percentage of exploration of the old object for the CRH-pretreated compared with the ACSF group (p<0.001). As well, *post hoc* testing revealed that animals that received intra-PFC Rp-cAMPS + CRH explored significantly more the old object compared to the CRH group (p<0.001) (Figure 19C). Furthermore, the 2-way ANOVA showed an effect of CRH ($F_{3,43}$=13.286, p<0.001), Rp-cAMPS ($F_{3,43}$=39.366, p<0.001) and Rp-cAMPS x CRH interaction ($F_{3,43}$=28.128, p<0.001) in the discrimination ratio between the old and recent object during the test phase of the temporal order memory test. Subsequent *post hoc* testing showed a significant decrease in the CRH pretreated rodents compared with the ACSF group (p<0.001), accompanied by a significant increase in Rp-cAMPs + CRH mice compared with animals that received intra-PFC CRH microinjections (p<0.001) (Figure 19D).

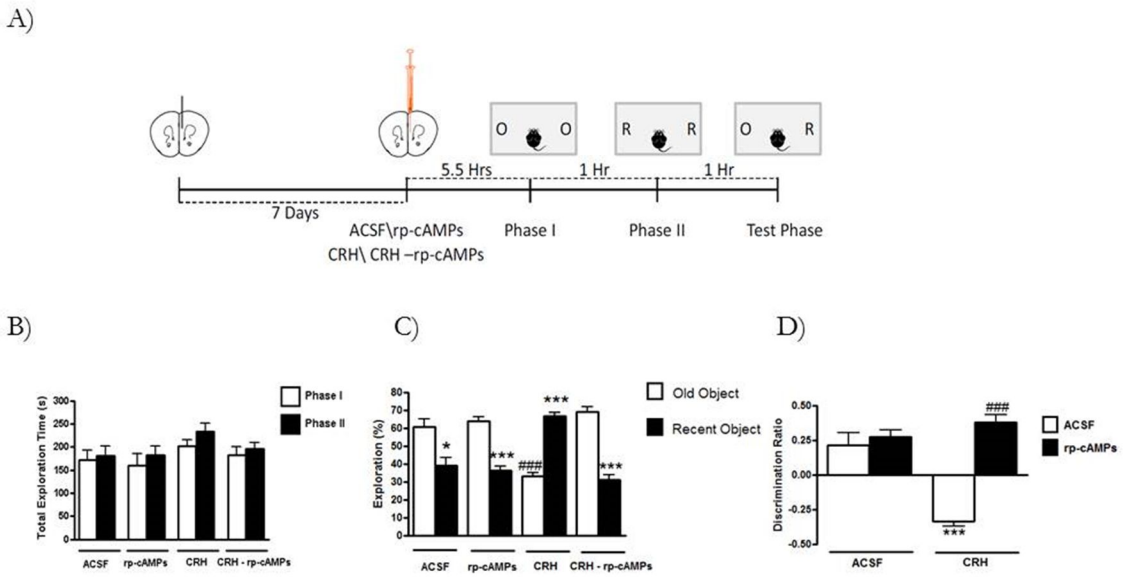

Figure 19: Effects of PKA blockade on the intra-PFC CRH-induced deficits in temporal order memory.

A) Experimental design. O= Old Object; R= Recent Object. B) Exploration times of the objects presented during the sample phases I and II of the temporal order memory test. C) Percentage of exploration of the old and recent objects presented during the test phase of the temporal order memory test. * Significantly different within the same group, $p<0.05$. *** Significantly different from the percentage of exploration of the old object within the same group, $p<0.0001$. ### Significantly different from the percentage of exploration of the old object in the ACSF group, $p<0.001$. D) Discrimination ratio between the old and recent objects presented during the test phase of the temporal order memory test. ** Significantly different from the ACSF group, $p<0.001$. ## Significantly different from the CRH-ACSF group, $p<0.001$.

IV.9.2. Effects of PKA blockade on the intra-PFC CRH-induced deficits in reversal learning

In addition, the same strategy was used to assess the role played by the PKA in the intra-PFC CRH-induced deficits in reversal learning. Thus, animals with a cannula implanted in the PFC underwent the training and retention sessions of the reversal learning test. On the 3rd day, they received intra-PFC microinjections of ACSF, Rp-cAMPS, CRH or Rp-cAMPs + CRH, and 6 hours later were tested in the reversal session (Figure 20A). All animals exhibited a significant reduction in the number of trials to reach criteria between the training and retention sessions (unpaired *t* test, $t_{(47)}=6.887$, $p<0.0001$) (Figure 20B). Furthermore, the 2-way ANOVA revealed a significant effect of Rp-cAMPs ($F_{(3,44)}=5.907$, $p<0.05$), CRH ($F_{(3,44)}=7.798$, $p<0.01$) and CRH x Rp-cAMPs interaction ($F_{(3,44)}=7.465$, $p<0.01$) in the number of perseverative errors made during the reversal session.

Subsequent *post hoc* testing showed a significant increase in the animals that received CRH compared with the ACSF group ($p<0.001$), accompanied by a significant decrease in the Rp-cAMPs + CRH compared to the CRH animals ($p<0.001$) (Figure 20C). Additionally, the 2-way ANOVA revealed a significant effect of Rp-cAMPs ($F_{(3,44)}= 25.798$, $p<0.001$), CRH ($F_{(3,44)}=15.054$, $p<0.001$) and Rp-cAMPs x CRH interaction ($F_{(3,44)}=6.590$, $p<0.05$) in the trials to reach criteria during the reversal session. Subsequent *post hoc* testing showed a significant increase in the CRH-pretreated mice compared with the ACSF group ($p<0.001$) accompanied by a significant decrease in rodents with Rp-cAMPs + CRH microinjections compared with the CRH group ($p<0.001$) (Figure 20D).

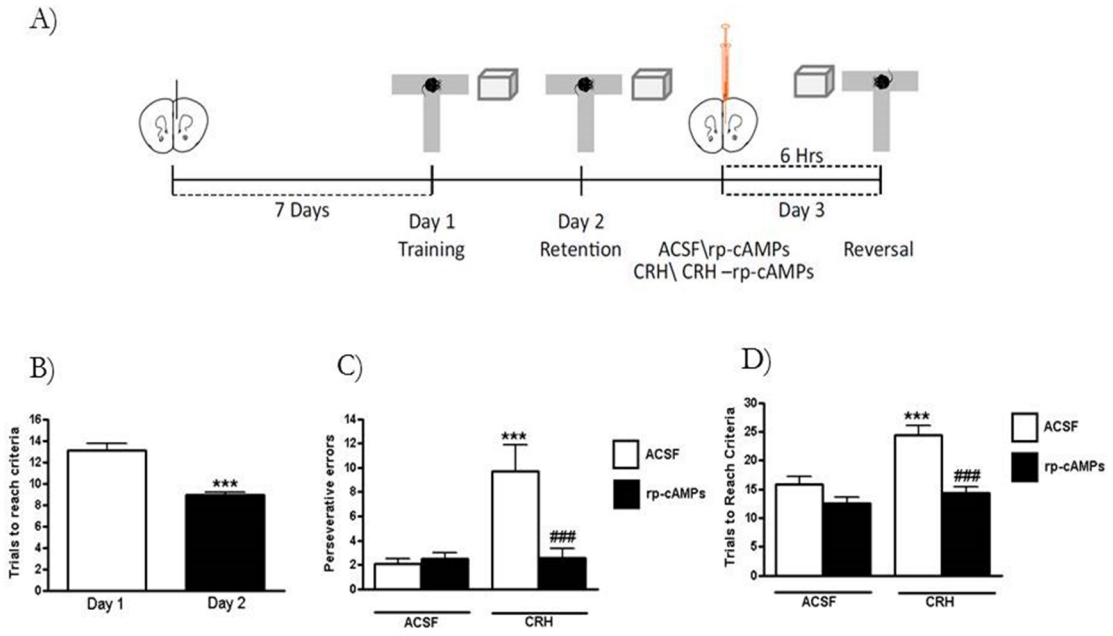

Figure 20: Effects of PKA blockade on the intra-PFC CRH-induced deficits in reversal learning.

A) Experimental design. B) Trials to reach criteria during the training and retention session. *** Significantly different from the number of trials to reach criteria on day 1, $p<0.0001$. C) Number of perseverative errors during the reversal session. D) Trials to reach criteria during the reversal session. ### Significantly different from animals that received CRH + ACSF microinjections, $p<0.001$. *** Significantly different from ACSF-pretreated rodents, $p<0.001$.

V. DISCUSSION

Modern society constitutes a highly dynamic scenario pervaded by cultural, social, emotional and biological events that eventually threat our lifestyle and have a clear impact in our physiology and behavior. As a result, we are exposed to different stressors, ranging from mild to robust, in a sporadic fashion during our everyday activities. In fact, several work placements combine high stress levels together with great responsibility, strongly requesting the executive control of behavior and cognition (Gunby, 1981; Jakobsson and Gustavsson, 2014; Proctor et al., 1996). Evidently, such circumstances place a great demand in our coping strategies, constantly requiring cognitive flexibility and executive functions, and trigger a set of adaptive responses that ultimately allow us to deal with these stressors in a more or less successful way. Thus, the current research project presents a robust set of data that convincingly positions the CRH-CRHR1 system as an essential player in the executive dysfunction that follows after acute stress exposure, thereby providing a molecular mechanism through which these effects take place. Therefore, it reveals a potential pharmacological target that could aid in the development of active measures to counteract the deleterious effects of stress on PFC-related processes.

V.1. Acute stress disrupts PFC-mediated cognition

Despite the fact that the effects of stress on cognition have been widely studied, it is still highly relevant to establish whether a single episode of stress is also able to induce such remarkable impairment. The current results elucidate the effects of a single 5-minute episode of social defeat on temporal order memory and reversal learning. Hence, whereas control animals display a higher exploration of the old object versus the recent object, stressed animals no longer exhibit such performance in the temporal order memory test. In fact, they seem to explore more the recent object. The effects of stress on temporal order memory have already been addressed in the literature, showing that early life stress experienced during the perinatal period affects temporal order memory in adulthood, through alterations in the dopaminergic system within the PFC (Lejeune et al., 2013; Proctor et al., 1996). As well, impairments in episodic-like memory have been observed after an acute stressor (Kart-Teke et al., 2006). However, such study exhibits a general impairment in which it is not possible to discern the cognitive domain in which the dysfunction lies. In this regard, the data obtained in the present project demonstrates that a

single episode of social defeat stress impairs the ability to correctly discriminate objects according to their relative recency.

The temporal order memory test constitutes a behavioral task that has been shown to depend on the integrity of the PFC networks (Barker et al., 2007). Naturally, the correct performance during the testing phase depends entirely on the exploration during acquisition phase. The effects of acute stress on the exploratory activity of rodents have been determined elsewhere in the literature (Efimenko et al., 2005). For instance, a 2 hour period of restraint stress induces decreases in locomotor activity 2 and 3 months later (Mamczarz et al., 2010). Similarly, another study showed acute stress-induced reductions in motor activity in an open field 15 minutes after the onset of the stressor (Ferretti et al., 1995). Likewise, 1 hour of immobilization leads to significant decreases in the exploration of the open field (Plaznik et al., 1993). However, it is important to stand out that the deficits cited in those studies refer mainly to locomotor activity elicited in the open field under standard illumination conditions. In the present research project, object exploration was used as a measure of exploratory activity, instead of locomotor performance. In addition, lower illumination conditions were employed (8-10 lux in the centre) and smaller open fields were used in order to promote object exploration. Furthermore, a longer time period was used between the stress session and the sample phase I. Taken together, the data obtained show no differences in object exploration between stressed and non-stressed animals. Additionally, no differences exist between sample phase I and sample phase II for each group of animals, indicating that both undergo similar exploration rates on each phase. This allows to discard any potential bias caused by a preference towards the objects presented on each phase. Altogether, the phenotype observed suggests that both groups explore similarly during the first 2 sample phases, encoding the same amount of information. Thus, it is possible to conclude that the deficit observed on the testing phase after stress reflects a temporal order memory impairment.

Besides temporal order memory, another cognitive test was employed to assess the effects of acute stress on PFC-mediated cognition. Several lines of evidence have established the role played by the PFC networks in reversal learning paradigms (Evans and Hampson, 2015; Hovens et al., 2014; McGaughy et al., 2014). The reversal learning paradigm employed in the current study is divided in three different sessions: training session, retention session and reversal session. Basically, in order to successfully assess PFC-dependent cognitive flexibility during the reversal session, animals need to easily acquire

and strongly retain a response pattern, in this case taking the arm of the T-maze leading to their homecage. As a matter of fact, the present results show that mice reach a stable performance in approximately 11 trials, suggesting that the task at hand does not represent a difficult challenge for them. Furthermore, a significant decrease in trials to reach criteria was observed between the training session and the retention session, with approximately 8 trials (the minimum possible) and with virtually no mistakes. Such phenotype clearly indicates a robust consolidation of the behavioral response assessed throughout the test and establishes a solid ground to assess cognitive flexibility. Posteriorly, during the reversal phase, different parameters were employed to evaluate this PFC-related function. On one hand, the number of perseverative errors, or mistakes executed before making the first correct response, constitutes a reliable measure of cognitive flexibility, as it elucidates the subjacent difficulty to switch a behavioral response for an opposite pattern despite the negative feedback received from the environment (Aizawa et al., 2012; Butts et al., 2013; Riley et al., 2011). Stressed animals display a significant increase in this measure compared with control animals, suggesting a greater difficulty to inhibit a predominant response and replace it for an opposite one. A second measure employed to assess cognitive flexibility was the number of trials to reach the cut-off criteria, a parameter that not only evaluates the ability to switch a predominant behavior, but also the stability of such behavioral change across time (Parker et al., 2012). The data obtained show that whereas control animals achieve this quite fast, stressed animals take longer to reach this criterion, indicating that the reversal learning test becomes a more challenging scenario after a single episode of social defeat. Hence, these findings strongly suggest that acute stress disrupts cognitive flexibility. The effects of acute stress in this PFC-related task have been previously explored in the literature. For instance, it has been shown that 15 minutes of mild tail-pinch stress immediately impair performance on a similar task (Butts et al., 2013). Those results, together with the present data, suggest that a single episode of stress induces deficits in cognitive flexibility that remain up to 8 hours after the onset of the stressor, suggesting the potential involvement of stress-induced genomic effects.

The effects of chronic stress on cognitive performance have been extensively addressed in the literature, together with its role in the development of psychiatric disorders such as depression, post-traumatic stress disorder and anxiety disorders, among others (Correa et al., 2014; Dieleman et al., 2015; Harris et al., 2014; Liu et al., 2015; Moench and Wellman, 2014). In this regard, chronic stress has been shown to have a strong effect upon cognitive processes including spatial memory, spatial recognition and attention (Matuszewich et al.,

2014; Xu et al., 2014), functions strongly supported by hippocampal networks. Thus, such chronic stress-induced dysfunctions are usually accompanied by alterations in hippocampal structure and physiology (Joo et al., 2014; Lehner et al., 2015; MacKenzie and Maguire, 2015). Concerning PFC-related functions, the situation doesn´t seem to be that clear. For instance, a recent study was able to demonstrate an absence of effects on executive functions in a population of clinical burnout patients (Oosterholt et al., 2014). On the other hand, a significant reduction in size, together with alterations in dendritic spines, have been observed in the PFC after chronic stress (Arnsten et al., 2015; Henckens et al., 2015; Moench and Wellman, 2014). Similarly, important decreases in the connectivity between the amygdala and the cingulate cortex have been reported in stressed subjects, suggesting a mechanism responsible for the emotional dysregulation observed after chronic stress (Golkar et al., 2014).

Besides such findings, additional studies have also determined the strong impact caused by a single stress episode. Relevant studies in this regard have been made and pertinent findings have been obtained. For example, humans undergoing an acute stress protocol exhibit increases in risk seeking, having a strong impact on economic decision-making (Buckert et al., 2014). Also, it has been demonstrated that acute stress triggers dynamic shifts in network balance, allowing the organism to reallocate its neural resources to a salience network, promoting fear and vigilance according to cognitive demands (Hermans et al., 2014). Furthermore, acute stress has been shown to alter the responsiveness of the reward system via cortisol (Oei et al., 2014). In addition, in-hospital acute stress is associated with a higher cognitive impairment that persists up to 12 months later (Davydow et al., 2013).

Regarding PFC-dependent cognition, the picture is far from complete. For example, ultrastructural analyses in animals have revealed an increase in the amount of docked vesicles and small excitatory synapses in the PFC after acute stress, leading to an overexcitation of the PFC networks and a potential executive dysfunction (Nava et al., 2014). Moreover, a single stress episode decreases d-serine levels in the PFC, giving rise to impairments in memory consolidation and prepulse inhibition (Guercio et al., 2014). Furthermore, it is known that a single episode of stress is able to alter PFC function leading to relevant deficits in fear inhibition in humans (Raio and Phelps, 2015). Surprisingly, a study showed an absence of effects on set-shifting accompanied by a facilitation of reversal learning after 30 minutes of restraint stress (Thai et al., 2013). Interestingly, the findings

presented in this thesis show that a single episode of social defeat stress induces a significant impairment in reversal learning 8 hours later. The differences between both outcomes can be ascribed to the stress paradigms employed and the time windows used to assess cognitive performance. Thus, whereas the restraint protocol constitutes a moderate stressor that confines the animals to a reduced space for a specific amount of time, the social defeat procedure exposes the rodent to a strongly aversive stimulus that directly threatens its own survival. Therefore, the social defeat stress comprises a more robust stressor and hence triggers a stronger stress response in the animals, leading to differential effects of those observed after restraint stress (Meerlo et al., 2001; Razzoli et al., 2010). Furthermore, the stress response exhibits a clearly dynamic deployment over time, where GCs reach a peak at about 1 hr after the stressor and remain elevated until approximately 8 hrs later (Wagner et al., 2013). Consequently, cognitive assessment performed at different time intervals after the stressor can reveal effects that rely upon divergent mechanisms (Quaedflieg et al., 2013). Accordingly, whereas effects observed immediately after stress can be caused by the release of stress-related hormones and the activation of their corresponding receptors, alterations registered 8 hours later can be triggered by effects upon genetic transcription (Wang et al., 2013).

V.2. Acute stress-induced PFC dysfunction is mediated by the CRHR1

As previously mentioned, several reports in the literature have elucidated the deleterious effects caused by both, acute and chronic stress, upon cognition. Along this line, different receptors have been regarded as key players mediating the cognitive dysfunction that follows after stress exposure. Concerning CRHR1, different experimental approaches have addressed the effects of stress on its modulation, together with its potential role in stress-mediated cognitive dysfunction. Thus, restraint stress induces an upregulation in CRHR1 expression in early-life stressed chickens (Goerlich et al., 2012). Another study showed differential sex-dependent changes in CRHR1 levels after acute stress exposure. Hence, female rats exhibited increases in CRHR1 mRNA in the amygdala, which correlated with enhanced anxiety-like behavior two hours after being subjected to the elevated plus maze. Males, on the other hand, did not show alterations in CRHR1 expression after acute stress (Zohar and Weinstock, 2011). The results obtained in the current research project show that a single episode of acute social defeat stress leads to significant increases in CRHR1 mRNA levels in different subregions of the PFC, including AC, PL and IL of male C57Bl6

mice. The apparent discrepancy between both studies can be explained by different factors. On one hand, each study employs different time points to assess stress-induced genetic modulation of the CRHR1. Therefore, whereas the former study measured CRHR1 levels 2 hours after stress, the present data are based on measurements performed 8 hours later. Genetic transcription constitutes a complex process involving a wide array of molecular mechanisms, all of which require considerate time windows in order to be fully expressed. Furthermore, signaling cascades affecting genetic transcription need additional time periods to completely reveal their effects. Consequently, longer timeframes allow the full expression of stress-induced genomic effects. Additionally, Zohar and Weinstock show alterations exclusively in females. Other lines of evidence have elucidated sex-related differences in the stress-evoked changes in genetic transcription, demonstrating that females exhibit stronger increases in gene expression after stress, suggesting an enhanced stress-sensitivity compared to males (Bohacek et al., 2015). Thus, it is possible that males require longer time periods than females in order to display stress-mediated genomic effects. Finally, both studies also differ in the regions analyzed. Consequently, whereas the former study focused on the amygdala, the present findings were registered exclusively in the PFC. Such divergence also indicates possible differential, stress-induced, region-specific effects upon CRHR1 modulation. However, in order to fully support this hypothesis, additional studies are still necessary.

Regarding the PFC, a research report observed an absence of effects upon CRHR1 levels within the PFC 4 weeks after unavoidable stress (Raone et al., 2007). Together with the present findings, this suggests that there seem to be differences between acute and chronic stress-induced alterations in CRHR1 expression in the PFC. However, the stress paradigms employed in both studies differ from each other. Therefore, to further confirm this idea, additional experiments are still required, that could include the measurement of changes in CRHR1 mRNA in the PFC of animals submitted to a chronic social defeat stress paradigm. Furthermore, intra-PFC alterations in CRHR1 expression have been reported in mice with high anxiety-related behavior (Sotnikov et al., 2014), suggesting the potential involvement of the CRH-CRHR1 system in the PFC dysfunction observed in anxiety disorders. Additionally, acute stress-induced changes in CRHR1 expression in the PFC have been observed in adult mice submitted to maternal separation in the early postnatal period (O'Malley et al., 2011). This indicates that early-life stress has an impact on the acute stress-induced alterations in CRHR1 in the PFC, and points out its potential relevance in the executive dysfunction caused by stress. However, the data obtained in the present project

strongly demonstrates the effects of a single stress episode in CRHR1 mRNA levels within the PFC.

As previously stated, perhaps the most studied neurochemical candidates regarding stress-induced cognitive impairment are GCs together with their corresponding GRs. As a matter of fact, it has been suggested that the slow genomic effects mediated by GR activation, in different brain regions, can affect contextual and higher cognitive aspects of behavioral performance that follow several hours after stress (Joels et al., 2013). As well, a crosstalk between GRs and other neurochemical systems has been proposed to mediate stress-induced cognitive dysfunction. Thus, it has been shown that following stress episodes, endocannabinoids interact with GCs, through GRs, on the cell surface of the BLA to promote BLA plasticity and memory consolidation, coordinating maladaptive emotional responses (Atsak et al., 2014). On the other hand, GRs have been implicated in the stress-mediated regulation of memory formation and modulation. In this regard, it has been demonstrated that acute stress promotes the formation of strong long-term memories via hippocampal GR activation (Finsterwald and Alberini, 2014). Such results are somehow opposite to the data obtained in the present research project, where an acute stressor is able to induce significant impairments in temporal order memory via CRHR1 activation. However, it is important to take into consideration that the two types of memory analyzed constitute different cognitive processes. Thus, whereas long-term memories are based in the maintenance and future retrieval of information, temporal order memory is defined as the ability to accurately discriminate the temporal order in which past events took place. Therefore, it is possible that acute stress can induce an enhancement of some cognitive functions at the expense of others, prioritizing the encoding and maintenance of biologically relevant information, to enhance future survival, versus more complex cognitive processes. Furthermore, it is also very likely that each of these effects can be mediated by different neurochemical systems.

Additionally, the role played by GRs in the PFC dysfunction associated with stress has also been addressed by different studies. For instance, chronic exposure to GR agonists impairs PFC-dependent decision-making. Such deficits are also accompanied by increases in dendritic spine density in the PL that decrease with acute corticosterone (Swanson et al., 2013). On the other hand, another study has shown a significant increase of GR expression and mRNA levels within the PFC after a single episode of stress (Wen et al., 2014). Similarly, in the present study, an increase in CRHR1 mRNA was observed 8 hours after a

single episode of acute social defeat stress. Taken together, these studies suggest a comparable intra-PFC upregulation of different stress-related-peptide receptors after acute stress.

Furthermore, in the current study, an intra-PFC specific deletion of the CRHR1 abolished the effects of acute stress on PFC-related cognitive functions. As a matter of fact, genetic deletion of the CRHR1 has been employed to assess the role played by this receptor in several behavioral and cognitive disorders different from executive dysfunction. For instance, it is known that CRHR1-KO rodents display lower anxiety levels compared to control animals (Chotiwat et al., 2010). Likewise, CRHR1 deficient animals show increased exploratory activity in the elevated plus maze and light-dark box, two widely used tests to assess anxiety-like behavior. However, these mice also present impairments in spatial recognition memory (Contarino et al., 1999). Even though anxiety-like behaviors were not directly assessed in the present study, the results obtained show no differences between PFC CRHR1 KO and WT in exploratory activity. In fact, both groups explored similarly across the different phases of the temporal order memory test, suggesting that the aforementioned effects of CRHR1 deletion on exploration are mediated by regions other than the PFC.

Particularly referring to the PFC, no studies addressing the effects of specific PFC deletion of the CRHR1 have been reported. As an interesting approximation, the conditional CRHR1 deletion, within the limbic forebrain, has been shown to significantly reduce remote, but not recent, associative and non-associative fear memories through impairments in the expression of GluR1-containing AMPA receptors (Thoeringer et al., 2012). Additionally, whereas the specific deletion of CRHR1 in forebrain glutamatergic circuits reduces anxiety and impairs neurotransmission in the amygdala and hippocampus, selective CRHR1 deletion in midbrain dopaminergic neurons increases anxiety-like behavior and reduces dopamine release in the PFC (Refojo et al., 2011b). These studies suggest a relevant crosstalk between the CRH-CRHR1 and other neurochemical systems within PFC networks, which could be responsible for the stress-induced cognitive and emotional alterations in PFC-mediated processes. However, they still lack region specificity. In fact, this constitutes the first study employing a PFC-restricted deletion of the CRHR1. Interestingly, the genetic strategy employed here gave rise to approximately a 50% reduction in CRHR1 expression, which is still within the lower range of the physiological

levels and encourages the potential use of pharmacological tools to achieve a similar decrease in CRHR1 activity.

Despite the fact that no studies have been performed assessing the role of CRHR1 in the PFC-dependent temporal order memory, there are reports in the literature elucidating the CRHR1-mediated effects on other memory processes that are PFC-independent. For instance, chronic stress-evoked disruptions in spatial memory and hippocampal CA3 dendritic morphology have been rescued using forebrain CRHR1 deficient mice, which in addition also present reductions in the chronic stress-induced downregulation of hippocampal GRs (Wang et al., 2011). Additionally, it has been shown that repeated exposure to novelty stress activates CRHR1 and delays the progression of Alzheimer´s disease-like cognitive decline, specifically concerning contextual fear memory and extinction performance (Scullion et al., 2013). Furthermore, CRHR1 deletion rescues the Alzheimer´s disease-like endocrine and behavioral phenotype observed in a genetic mouse model of this neurodegenerative condition (Guo et al., 2012). Therefore, the effects of stress, via CRHR1, in the cognitive decay that characterizes the progression of neurodegenerative diseases has been somehow elucidated elsewhere in the literature. Nonetheless, there seems to be a lack of studies addressing a direct link between the PFC CRHR1 and the acute stress-induced executive dysfunction. In this regard, an interesting approach indicated an association between CRHR1 polymorphisms and age-dependent impairments in working memory, a PFC-related process (Grimm et al., 2014). Similarly, another report showed that specific CRHR1 polymorphisms determine early-life stress-evoked effects on working memory performance (Fuge et al., 2014). The present findings constitute, not only stimulating evidence elucidating the role of intra-PFC CRHR1 on the acute stress-induced disruptions in other memory processes, but also novel data showing that this receptor seems to play a major contribution in the cognitive rigidity that follows after a single episode of stress. In other words, this study suggests that a single episode of social defeat stress leads to CRHR1 activation in the PFC, which eventually disrupts higher cognitive functions that allow an organism to flexibly change behavior according to external circumstances and to precisely organize temporal information. Such outcomes strongly posit the CRHR1 as a potential therapeutic target to attenuate stress-induced executive dysfunction.

Analogous strategies have been employed in the literature concerning other stress-related candidate systems in diverse brain regions (Jacobson, 2014; Jeanneteau et al., 2012b;

Vincent and Jacobson, 2014). Comparable approaches involving PFC networks have also been performed. For instance, mice lacking the MR in hippocampal-amygdala-PFC circuitry exhibit robust deficits in social discrimination and decision-making (Ter Horst et al., 2014), indicating the relevance of stress-related-peptide receptors in PFC-mediated cognitive functions. Another study using forebrain GR knockout mice, encompassing the PFC, found increases in depression-like behavior in the forced swim and sucrose preference tests (Solomon et al., 2012). Interestingly, a similar approach has been employed inducing PFC-restricted knockdown of GR. Mice that underwent this procedure exhibited an increased behavioral sensitivity to imipramine treatment in the forced swim test (Hussain and Jacobson, 2015). This indicates that different receptors of stress-related peptides seem to play a key role in the vulnerability of the PFC-networks to stress, and thus constitute attractive targets to develop novel strategies to counteract the acute stress-induced executive dysfunction. Despite the fact that several lines of evidence point to a strong involvement of GRs in the stress-induced executive dysfunction, literature addressing the contribution of the CRHR1 is still scarce.

V.3. Intra-PFC CRH administration mimics acute stress-induced PFC dysfunction

Given the relevant role played by the stress-related peptide CRH in the fast initiation of the stress response, several lines of evidence have also explored the effects of CRH on cognition, building a link between stress and cognitive impairment. Regardless of the pertinence and importance of such findings, reports in the literature directly linking intra-PFC CRH with the executive functions exerted by the PFC networks are still scarce. In this respect, the present results clearly indicate that intra-PFC CRH micro-infusions exert a deleterious effect in temporal order memory and reversal learning 8 hours after administration. Moreover, such phenotype strongly resembles the one observed 8 hours after a single episode of acute stress. Foremost, CRHR1 blockade abolishes the intra-PFC CRH-induced executive dysfunction, building a strong connection between acute stress, CRH release, CRHR1 activation and cognitive impairment. In this regard, in order to unravel the role played by the CRHR1 in the CRH-induced executive dysfunction, a non-selective CRH receptor antagonist was co-infused together with CRH in the PFC. It is important to stand out that CRHR2 is basically absent from PFC networks (Reul and Holsboer, 2002), thus supporting the notion that the observed effects are conveyed by the

CRHR1 receptor. In addition, the improvement in PFC-mediated cognition after CRHR1 blockade mirrors the one observed after intra-PFC CRHR1 deletion.

Concerning the temporal order memory test employed in the current study, increases in anxiety-like behavior can easily disrupt performance and affect the final readout. Actually, the effects of intra-PFC CRH microinjections on anxiety have been widely explored in the literature. Nonetheless, there seems to be heterogeneity in the results observed that vary depending on dose and additional factors. For instance, whereas one study found no changes in anxiety-like behavior after intra-PFC CRH-administration in unstressed animals (Magalhaes et al., 2010), another showed increases using the same procedure in acutely and repeatedly stressed rodents (Jaferi and Bhatnagar, 2007). In a similar fashion, intra-PFC CRH micro-infusions at different concentrations have shown an anxiogenic-like effect in the elevated plus maze that is attenuated by CRHR1 blockade in the same region (Miguel et al., 2014). Despite the fact that the present data do not include a direct measure of anxiety-like behavior after CRH microinjections and CRHR1 blockade, no differences were observed in exploratory activity during the two initial phases of the temporal order memory test. Several lines of evidence have shown the relationship existing between anxiety states and object exploration (Green et al., 2013; Vogt et al., 2014). Thus, CRH microinjections in the PFC do not seem to have an overall impact on anxiety-like behavior. In fact, this absence of effects on anxiety does not seem to depend on the concentration employed, because the two different concentrations used in our experiments exhibited comparable exploration rates. Most likely, the discrepancies between the aforementioned studies and the present findings rely on the time frame taken to assess behavior. Whereas in the former reports, exploratory activity was evaluated immediately after drug administration, the current results were obtained up to 5 hours after the pharmacological intervention (sample phase I). Notwithstanding the fact that the role of CRH on stress-related mnemonic processes has been already elucidated, there is no evidence regarding its contribution to temporal order memory processes. Furthermore, the data obtained in the present study show a significant increase in the percentage of exploration of the recent object versus the old object in the CRH-pretreated animals. It has been demonstrated that acute CRH release can enhance memory processes (Chen et al., 2012). In fact, it is possible that acute increases in CRH can actually reinforce recent memory traces, thus improving the encoding and maintenance of biologically salient information and disrupting other higher cognitive functions. Such physiological reaction could actually help to explain the observed phenotype, in which the memory trace closer in time to the CRH administration (old

object), is so strongly encoded that animals tend to explore more the object presented farther in time from the CRH microinjection (recent object), disrupting the ability to properly discriminate the temporal order in which these events took place.

In addition, the current findings also show impairments in reversal learning 8 hours after intra-PFC CRH administration. The effects of CRH microinjections in cognitive flexibility have already been explored elsewhere in the literature. However, the role played by this stress-related peptide in reversal learning is still not very clear. For instance, whereas intracerebroventricular CRH infusions compromised this cognitive function, intra-locus coeruleus microinjections had an opposite effect, facilitating cognitive flexibility (Snyder et al., 2012). Such findings, together with our current data, suggest a differential, region-specific, effect of CRH administration on reversal learning. Thus, the present study constitutes highly relevant evidence indicating that acute CRH administration in the PFC, gives rise to important alterations in the ability to switch a behavioral response for an opposite pattern, according to environmental feedback. As well, the actual results show that a single microinjection of CRH in the PFC leads to a trend towards a significant increase in the number of perseverative errors. Likewise, such pharmacological intervention also gives rise to a significant increment in the trials to reach criteria during the reversal phase. This behavioral pattern strikingly resembles the one observed after acute social defeat stress. Furthermore, CRHR1 blockade in the same brain region surprisingly rescues CRH-induced deficits in a similar fashion to the phenotype observed after intra-PFC CRHR1 deletion. Taking this into consideration, together with the fact that all experiments employed the same time windows after the experimental procedure, we hypothesize that acute stress leads to increases in CRH release within the PFC, which eventually activates CRHR1 receptors and unleashes an executive dysfunction that affects higher-order cognitive processes.

The role played by CRH in cognitive performance has already been addressed elsewhere in the literature. For instance, it has been established that CRH is released from hippocampal axon terminals during stress, exerting time and dose dependent effects on learning and memory, through the modulation of synaptic function and plasticity. Thus, whereas physiological levels of CRH, acting over seconds to minutes, enhance memory processes, exposure to high stress-peptide levels results in spine retraction and loss of synapses (Chen et al., 2012). Furthermore, increases in hippocampal CRH together with memory dysfunctions are observed in adult rodents submitted to early life stress. In fact, blockade

of CRHR1 signaling significantly attenuated the hippocampal synaptic dysfunction and the memory deficits. In addition, such early life stress-induced memory impairments could also be mimicked by forebrain CRH overexpression (Wang et al., 2014; Wang et al., 2013). Additionally, a knockin mouse model of Alzheimer´s disease exhibits increases in CRH levels along different brain regions, accompanied by elevated resting levels of corticosterone, increased anxiety-like behavior and memory impairments (Guo et al., 2012). Moreover, intra-amygdalar CRH overexpression induced after fear conditioning leads to increases in the expression of the conditioned emotional memory, thus modulating conditioned anxiety-like behaviors and contributing to the inappropriate regulation of emotional memories (Sink et al., 2013). Despite the high number of studies demonstrating the deleterious effects of CRH upon cognitive performance, few of them have focused on the PFC networks and the potential involvement of this peptide in the stress-induced executive dysfunction. In this regard, it has been shown that a single episode of restraint stress increases CRH mRNA levels in the PFC. Additionally, CRH administration in PFC neurons enhances CRHR1 expression, thus suggesting a potential involvement of the CRH-CRHR1 system in the acute stress-induced executive dysfunction (Meng et al., 2011). As well, it is known that the activation of CRH neurons in the medial PFC strongly contributes to the loss of inhibitory control that characterizes many addictive and impulsive behaviors (Zorrilla et al., 2014). In fact, a study performed in post-mortem tissue of depressed suicides, found elevated levels of CRH in the dorsolateral and ventrolateral subdivisions of the PFC, pointing out once more the relevance of this peptide in the behavioral loss of control related to PFC dysfunction (Merali et al., 2006).

Naturally, besides the CRH-CRHR1 system, there are also many other stress-related neurochemical systems whose effects upon cognition have been widely elucidated. Therefore, in order to accurately identify the neurochemical candidates responsible for the acute stress-induced cognitive impairment, it is essential to refer as well to other stress-related peptides that play a fundamental role in the initiation and maintenance of the stress response. In this regard, perhaps the most studied target has been the GC system. Hence, several lines of evidence have focused on the GCs as key mediators of the acute stress-induced cognitive deficits. Accordingly, a study performed in humans showed a strong association between higher cortisol levels released during acute stress and poorer cognitive performance on declarative memory tasks (Lasikiewicz et al., 2013). Furthermore, acute stress has been shown to induce a significant attenuation in LTP in CA1, together with relevant increases in corticosterone levels in the same region. Interestingly, hippocampal

sections exposed to corticosterone exhibit the same reduction in LTP observed after acute stress (Takeda et al., 2012). In addition, it has also been demonstrated that acute stress leads to important impairments in memory retrieval that are mimicked by the administration of GC (Schutsky et al., 2011a), suggesting once more the key involvement of GCs in the acute stress-induced cognitive dysfunction. Furthermore, it has been shown that both, intra-PFC administration of corticosterone and acute stress, give rise to increases in dopamine efflux together with impairments in working memory, indicating the relevance of stress-related peptides in the acute stress-induced executive dysfunction (Butts et al., 2011b). However, the situation is far from simple. As a matter of fact, different research reports have elucidated many acute stress-evoked cognitive dysfunctions that emerge independently of the GC system. For instance, it has been demonstrated that acute stress dynamically reduces nectin-3 levels in the hippocampus via CRHR1 signaling. This is followed by spatial memory deficits and dendritic spine loss. Interestingly, such effects are not mediated by GC receptors (Wang et al., 2013). Moreover, concerning PFC-related tasks, a study showed that a single episode of restraint stress facilitated reversal learning 30 minutes later, in a GR and MR independent fashion, suggesting the involvement of additional stress-related peptides (Thai et al., 2013). Similarly, another research report showed acute stress-induced deficits in decision-making that were not mimicked by the treatment with physiological doses of corticosterone, pointing once more to mechanisms mediated by other neurochemical candidates (Shafiei et al., 2012). The present findings, together with the aforementioned literature, point to acute stress-evoked alterations in executive functions that emerge via the activation of the CRH-CRHR1 system.

V.4. Acute stress and CRH activate protein kinase A signaling in the PFC

Unraveling the contribution of a neurochemical system to the acute stress-induced PFC dysfunction should encompass much more than just identifying the circuits, ligands and receptors responsible for such deleterious effects. As a matter of fact, once receptors are activated by their corresponding ligands, they undergo structural modifications that are eventually translated into physiological reactions, involving either changes in membrane permeability or the stimulation of signaling cascades that recruit several molecular players, which finally affect different cellular processes. As already mentioned, according to the current findings, the CRH-CRHR1 system seems to be a key effector in the acute stress-

induced executive dysfunction. However, there is still a gap between the activation of the CRHR1 and the resulting cognitive impairment. Thus, it is highly relevant to explore the molecular mechanisms that follow CRHR1 activation in the PFC, and strongly contribute to its impairment. The present findings make an important contribution to this field, indicating that both, acute stress and CRH, activate the PKA molecular cascade within the PFC networks. Altogether, the mentioned evidence strongly suggests that the acute stress-induced activation of the PKA cascade, via CRHR1, in the PFC exerts a significant cognitive impairment.

In this regard, several lines of evidence have demonstrated that CRHR1 stimulation can actually activate different signaling cascades in a region-specific, cellular context-related and activity-dependent manner. Thus, CRH administration in hippocampal slices induces coupling of CRHR1 to G_s, G_q, and G_i, clearly indicating the diversity of signaling pathways linked to CRHR1 stimulation in the mouse (Blank et al., 2003). Furthermore, another study showed that CRH leads to G_i and G_q protein activation, hence decreasing cAMP production and increasing phospholipase C-beta 3 and inositol triphosphate, with a concurrent enhancement of intracellular Ca+ (You et al., 2012). As well, CRH-mediated effects upon genetic transcription, via the CRHR1-dependent activation of phospholipase C and protein kinase C, have been observed in hippocampal cells. Surprisingly, neither adenylyl cyclase nor PKA inhibitors seem to affect CRH-induced increases in genetic transcription (Sheng et al., 2012). Interestingly, CRHR1 activation in hippocampal cells has also been shown to control the expression of the inducible serum/glucocorticoid-inducible protein kinase-1 in an adenylyl cyclase and PKA-dependent manner (Sheng et al., 2008). Additionally, it has been suggested that the CRH-mediated stimulation of CRHR1, with the concurrent activation of the PKA-CREB pathway, leads to a neuroprotective effect against the progression of Alzheimer´s disease (Bayatti and Behl, 2005). Concerning the molecular pathways stimulated by stress, it has been shown that acute stress is able to induce deficits on memory retrieval via the activation of $G_{i/o}$-coupled $ß_2$ adrenergic receptors, which lead to decreases in cAMP levels. Interestingly, increases in cAMP signaling rescue such impairments (Schutsky et al., 2011b). Furthermore, another study demonstrated that acute stress can actually enhance working memory processes through increases in the surface expression of NMDA and AMPA receptors in the PFC. These effects depend on the activity of the glucocorticoid-inducible kinase and the consequent stimulation of Rab4 (Yuen et al., 2011). Similarly, hippocampal slices of rodents submitted to an acute stressor exhibit an NMDA- and PKA-dependent insertion of Ca+ permeable AMPA receptors into

synapses, suggesting a mechanism through which acute stress can actually enhance cognitive performance (Whitehead et al., 2013). Taken together, those studies suggest that the PKA signaling cascade seems to be somehow involved in the cellular effects caused by CRHR1 activation. In addition, such molecular pathway also seems to be involved in the effects of acute stress upon cognition, via GRs and other neurotransmitter systems, at least in the hippocampus. However, the effects evoked by the stimulation of this signaling cascade upon hippocampus-mediated cognition appear to subserve a beneficial function. Moreover, approaches integrating the signaling cascades activated by acute stress and the CRH-CRHR1 system in extra-hippocampal circuits are still missing. Therefore, the obtained data provide an important contribution to this topic and establish PKA as a potential molecular candidate mediating the executive dysfunction that follows after acute stress exposure.

As well, literature elucidating the effects of CRHR1 activation upon the activity of the different subunits of the PKA is still scarce. The present results constitute a relevant addition to this subject, showing that both, acute stress and a single intra-PFC CRH microinjection, induce a similar activation of the PKA signaling pathway in the PFC, indicated by a decrease in the interaction between the regulatory and catalytic subunits, accompanied by an increase in the binding between the catalytic subunits and the CREB transcription factor. Such findings present a potential molecular signature responsible for the executive dysfunction observed after acute stress, via CRH-CRHR1. The effects of CRHR1 stimulation on genetic transcription, via the activity of the PKA, have been already established in the literature using cerebellar cultures. Thus, the gene transfer of a mutated regulatory subunit of PKA, lacking cAMP binding sites, completely suppresses the CRH-dependent transcription of c-fos, without affecting the increase in cAMP levels (Barthel and Loeffler, 1993). Furthermore, stress has been shown to modulate the activity of PKA via GCs. For instance, GC administration leads to a selective decrease in the levels of the regulatory subunit of PKA without altering its catalytic subunit. Such findings are accompanied by reductions in CREB phosphorylation, elucidating a link between stress, PKA activity and genetic transcription (Liu et al., 2012b; Sacai et al., 2014). Conversely, another study has demonstrated that dexamethasone can paradoxically stimulate cortisol release through a GR-mediated direct effect on the catalytic subunit of PKA (Louiset et al., 2009). In fact, there are in vivo and vitro evidences suggesting that GRs associate with the catalytic subunit of PKA, directly regulating PKA´s activity (Doucas et al., 2000).

Once the regulatory and catalytic subunits of PKA are uncoupled, the catalytic subunit is translocated to the nucleus and there induces the phosphorylation of the CREB transcription factor to regulate genetic transcription. According to the current findings, a single episode of defeat stress leads to significant increases in pCREB in the PFC. Such pattern clearly contrasts with the effects observed after chronic stress, which has been shown to evoke a decrease in pCREB levels in different brain regions, including CA1, CA2, CA3, paraventricular nucleus of the thalamus, amygdala and anterior cingulate area, that return to normal levels after a recovery period (Lin et al., 2009). As well, a diminution in pCREB levels were registered in the hippocampus of rats submitted to a paradigm of chronic stress involving olfactory bulbectomy (Jindal et al., 2015; Lin et al., 2009). Similar outcomes have also been obtained employing chronic social defeat stress protocols (Fan et al., 2015). Regarding the PFC, a study reported significant decreases in the number of cells expressing pCREB within the PFC of rats submitted to chronic stress (Lin et al., 2008). These observations suggest a discrepancy in the molecular effects caused by acute and chronic stress, possibly due to compensatory mechanisms triggered during chronic stressors. However, additional studies are required to further confirm this hypothesis. Furthermore, in agreement with the current results, increases in pCREB have been observed within the dentate gyrus and CA3 after acute sleep deprivation in rats (Azogu et al., 2015). Additionally, another study has suggested an age-dependent hippocampal activation of cellular and molecular mechanisms after acute stress. Hence, while juvenile rats exhibit increases in pCREB levels in the hippocampus after restraint stress, adult rats do not display the same effect (Chen et al., 2006). The apparent divergence between this data and the present results can be explained by the different stress paradigms employed and the different regions analyzed. Concerning the role of the CRH-CRHR1 system in the stress-induced CREB phosphorylation, there is evidence showing that the stress-evoked increases in pCREB, within the lateral septum and nucleus accumbens, are abolished after pretreatment with a CRHR1 antagonist (Kreibich et al., 2009). Similarly, employing a contextual fear conditioning paradigm, the oral administration of a CRHR1 antagonist leads to relevant decreases in contextual freezing, accompanied by reductions in pCREB levels in the lateral and basolateral amygdala (Hubbard et al., 2007). This phenotype also offers an interesting link between stress, CRHR1 activation, CREB phosphorylation and behavioral output, emphasizing the relationship between the CRH-CRHR1 system, the PKA signaling cascade, gene transcription and behavior. The present findings demonstrate that both, acute stress and CRH, exert deleterious effects upon PFC-mediated cognition

and activate the PKA molecular pathway culminating in CREB phosphorylation, thus showing agreement with the aforementioned studies.

As explained, besides the phosphorylation of the CREB transcription factor, stress and CRH have also been shown to stimulate additional signaling cascades following the activation of PKA. In fact, perhaps one the most studied molecular candidates also mediating many of the cellular and cognitive effects caused by stress, is ERK. Interestingly, the increase in PKA activity was followed by increases in CREB phosphorylation, which were not accompanied by increases in pERK levels. This suggests a selective triggering of molecular candidates leading to executive dysfunction. In this regard, decreases in the levels of ERK phosphorylation in the PFC have been observed after 5 weeks of chronic unpredictable stress, accompanied by impairments in the Morris water maze and the novel object recognition task (Liu et al., 2014). As well, increases in pERK occur in the CA1 region during a fear conditioning/extinction protocol employing repeated extinction trials. Similar effects were registered in the PFC after extinction retrieval (Ishikawa et al., 2012). Along this line, it has been established that CRHR1 mediates genetic transcription through the ERK 1/2 pathway (Zhao et al., 2013). In addition, employing hippocampal cells, the CRH-CRHR1-mediated activation of ERK 1/2 has been shown to involve a PKA and Rap1-dependent stimulation of cAMP and B-Raf (Bonfiglio et al., 2013). Thus, the results obtained in the present study suggest specificity in the molecular pathways activated by acute stress, via CRHR1, in the PFC. However, additional studies addressing different signaling cascades and time windows are still necessary in order to fully support this hypothesis.

V.5. CRH-induced PFC dysfunction is mediated by PKA

To further close the gap between acute stress, the CRH-CRHR1 system, PKA activity and executive dysfunction, we investigated whether PKA stimulation is really involved in the PFC impairments observed after acute defeat. The results obtained show that PKA blockade abolishes the intra-PFC CRH-induced executive dysfunction. PKA has also been demonstrated to be critically implicated in the cognitive impairments that characterize several psychiatric and neurological conditions. Accordingly, PKA activation is highly involved in anxiety, social interaction, aggression and learning. Thus, decreases in PKA activity in the amygdala lead to loss of excitatory synapses and severe neurobehavioral disabilities that are rescued by in vivo infusion of cAMP analogues (Jayachandran et al.,

2014). On the other hand, excessive PKA stimulation seems to underlie the deficits in working memory and hippocampal long-term depression that are exhibited by rats with juvenile-onset diabetes miellitus (Sacai et al., 2014). Additionally, the excessive activation of the PKA signaling pathway induced by stress, has also been implicated in the accompanying cognitive dysfunction in learning and memory, which is abolished by the genetic ablation of a PKA´s substrate phosphorylation site (Liu et al., 2012a; Sacai et al., 2014). Summarizing, increases in PKA activity seem to significantly disrupt the physiological activity of neural circuits, leading to important cognitive and behavioral alterations. Furthermore, the imbalance of additional signaling pathways has also been demonstrated to induce deleterious effects upon cognition, and thus contribute to pathological responses. Hence, several isoforms of PKC have been implicated as fundamental players in the maintenance and expression of the physiological processes that underlie long term memory. On the other hand, PKC disruption impairs non-associative memory in a paradigm of drug addiction, highlighting as well the role played by PKC in addiction-related memory, particularly in the transition from casual to pathological drug use (Howell et al., 2014). Furthermore, increases in the phosphorylation of the Ca+/calmodulin-dependent protein kinase II (CaMKII) coincide with a recovery of the motor deficits and cognitive impairment observed in an animal model of Parkinson´s disease (Yabuki et al., 2014a). As well, a restoration in the autophosphorylation of CaMKII, GluR1 and PKC, together with improvements in spatial reference memory and short-term memory, were observed after the pharmacological intervention of an animal model of Alzheimer´s disease (Moriguchi et al., 2013). The current findings, together with the aforementioned studies, suggest that PKA constitutes a key molecular candidate mediating proper cell functioning, and thus is an important cognitive modulator. Therefore, slight changes in its delicate balance can bring deleterious consequences for the organism, which might be prevented by the restoration of its normal levels using pharmacological tools.

Moreover, PKA activity has been strongly connected with PFC-related cognitive functions. Interestingly, under normal conditions, PKA suppression has been shown to underlie accurate performance in a working memory task in rodents. Furthermore, impairments in working memory together with increases in both, basal and working memory-related, PKA activity were observed in animals submitted to traumatic brain injury. Consequently, intra-PFC infusions of the PKA blocker Rp-cAMPS in animals with traumatic brain injury, improved working memory performance 24 hours later (Kobori et al., 2015b). Such results clearly match the present findings, in which CRH- and acute stress-induced increases in

PKA activity, within the PFC, are accompanied by deficits in temporal order memory and reversal learning. Foremost, this executive dysfunction is rescued by the intra-PFC administration of the PKA blocker Rp-cAMPS, indicating the role played by excessive PKA stimulation in PFC-mediated cognition, and postulating PKA as a potential therapeutic target to ameliorate acute stress-induced executive dysfunction. Hence, intra-PFC downregulation of PKA, CREB and pCREB, has been demonstrated in rats submitted to a chronic unpredictable mild stress paradigm (Chen et al., 2013). As a matter of fact, working memory deficits and decreases in pCREB levels in the PFC have been demonstrated after 6 months of chronic alcohol consumption followed by 1 or 6 weeks of withdrawal. Enhancement of CREB activity prior to testing improves working memory performance and increases pCREB exclusively in the PFC of withdrawn mice. Remarkably, intra-PFC infusion of a PKA activator mimics these effects (Dominguez et al., 2014). Furthermore, concerning PFC-related cognitive performance, different molecular candidates have been also identified as relevant mediators contributing to the expression of the executive dysfunction that accompanies different psychiatric disorders. For instance, an excessive phosphorylation of the CaMKII has been observed in the PFC of rats exhibiting attention-deficit/hyperactivity disorder (ADHD)-like behaviors. Interestingly, this molecular signature is reduced after the administration of methylphenidate, a therapeutic approach widely used to treat children with ADHD (Yabuki et al., 2014b). Similarly, decreases in the autophosphorylation of the CaMKII, and its substrate GluR1, have been identified in the PFC of rats with neonatal ventral hippocampus lesions, an animal model of schizophrenia characterized by alterations in prepulse inhibition, spontaneous locomotion and social interaction (Yabuki et al., 2013). Additionally, PKC overactivity in the PFC has been detected after sleep deprivation in an animal model of bipolar disorder. This molecular hallmark is also accompanied by hyperlocomotion and increased sleep latency. Importantly, PKC inhibitors attenuated these manic-like behaviors (Abrial et al., 2014).

As previously mentioned, the temporal order memory test is based on the exploratory activity of the rodents, and consequently any potential alteration in their behavior can have a strong impact in the outcome of this test. The present data show an absence of effects in exploratory activity after PKA inhibition during the first two phases of the test. Such results agree with others presented elsewhere in the literature (Barros et al., 2000). On the other hand, it has been shown that mice with decreased PKA signaling exhibit decreased exploration in the elevated-plus maze and open field (Davis et al., 2012). However, it is

important to mention that both mentioned tests evaluate locomotor activity in big spaces under high illumination conditions, whereas the temporal order memory test analyses object exploration in a more confined area under much lower illumination. Therefore, it is hard to establish direct comparisons between both approaches. In addition, the fact that similar exploration rates were observed for both objects during the first two sample phases for all groups, suggests an absence of a preference towards any of them. Regarding memory processes, previous studies have identified memory deficits after PKA inhibition (Arnsten et al., 2005; Bollen et al., 2014), which contrast with the results obtained in the present experiments. In order to further resolve this discrepancy, it is important to consider that both approaches address different types of memory, subserved as well by different brain regions. This, together with the current findings, suggests a region-specific and activity-dependent role for PKA in memory modulation. Nonetheless, additional studies are still required to support this hypothesis. Among the different subtypes of memory processes, the temporal order memory encompasses a particular set of high-order cognitive skills, including the consolidation, maintenance, retrieval, and most importantly, temporal organization, of past events. Surprisingly, despite the fact that the role of PKA in several hippocampal and PFC-dependent memory processes has been established, studies addressing the involvement of PKA in temporal order memory are still scarce. Thus, this set of experiments constitutes stimulating evidence in this regard, demonstrating as well that PKA blockade fully reverses the CRH-induced temporal order memory dysfunction.

Besides temporal order memory, the present data exhibit the contribution exerted by PKA to the deficits caused by intra-PFC CRH microinjections in reversal learning. The present findings suggest that increases in PKA signaling, through CRH, disrupt cognitive flexibility. These effects are abolished after a specific PKA inhibition in the PFC. This agrees with additional studies addressing the role exerted by PKA in cognitive flexibility. For instance, region-specific increases in PKA-R expression have been registered in the hippocampus during different stages of a spatial learning task involving a reversal component (Havekes et al., 2007). Naturally, enhancing PKA-R levels should also increase PKA-R to PKA-C binding, and thus decrease the amount of activated PKA-C. Therefore, these effects could eventually be translated into a total decrease in PKA activity. Consequently, it is possible to ensure that reductions in PKA activity are responsible for an accurate performance in a reversal learning task. Furthermore, the current study shows that PKA blockade does not induce any effects in reversal learning. Such findings differ from other studies, in which a genetically-induced deficiency in PKA signaling disrupted reversal learning in a Morris

Water Maze task without affecting fear conditioning (Rutten et al., 2011). Nonetheless, there are discrepancies between both experiments that need to be taken into consideration. The former study employed the Morris Water Maze, a behavioral test usually employed to assess spatial memory, and therefore hippocampus-dependent cognition, whereas the present approach used a cognitive test that does not possess such strong spatial component. In addition, whereas the former study was based on a permanent PKA disruption, the present experiment employed a transient, pharmacologically-induced, decrease in PKA activity with a corresponding behavioral assessment 8 hours later. Given the short time action of the PKA-inhibitor, it is expected that by the time reversal learning was evaluated, PKA activity was already back to normal (Dostmann, 1995). Hence, the observed effects on cognitive flexibility are possibly caused by a transitory increase in PKA signaling, evoked by intra-PFC CRH, which was prevented by the co-infusion of the PKA blocker. Consequently, these data indicates that PKA constitutes an essential molecular player mediating the effects of acute stress, via the activation of the CRHR1 in the PFC, in PFC-related cognitive processes.

VI. SUMMARY

The contemporary civilization constitutes a highly demanding environment, undergoing constant development and bringing permanent change to living conditions. Such circumstances strongly demand high levels of adaptability and flexibility, placing a great burden on the functionality of the PFC networks responsible for the executive control of cognition and behavior. Therefore, deciphering the neurochemical and molecular candidates responsible for the acute-stress induced executive dysfunction, will provide valuable tools in the development of novel strategies to improve quality of life in modern society. In this regard, the present findings established the CRH-CRHR1 system as a key mediator in the PFC-related deficits caused by acute stress. Thus, exposure to an acute stressor led to impairments in temporal order memory and cognitive flexibility, together with increases in CRHR1 mRNA levels within the PFC. In addition, deletion of the CRHR1 in the PFC ameliorated the effects of acute stress in these PFC-related tasks. Moreover, intra-PFC CRH microinjections mimicked the acute stress-induced deficits in PFC-dependent cognition. Additionally, both acute stress and intra-PFC CRH activated PKA and increased CREB phosphorylation in the PFC. Finally, intra-PFC blockade of PKA abolished the executive dysfunction caused by CRH.

Summarizing, the present findings suggest that upon an aversive situation, CRH is released in the PFC networks and binds to CRHR1 receptors, which eventually stimulate the G_{α} protein and increase cAMP levels. As a result, cAMP binds to the PKA-R and induces a disassembly of the PKA tetramer complex, causing a release of the PKA-C and its translocation into the nucleus. There, it binds to the transcription factor CREB, signalizing its phosphorylation and promoting genetic transcription that ultimately has a deleterious effect on the functionality of the PFC, leading to deficits in temporal order memory and reversal learning. Naturally, there are still gaps that must be addressed to fully give support to this hypothesis. For instance, it is crucial to further determine the genes that are upregulated after the activation of this molecular cascade and play a major role in the executive dysfunction observed afterwards. Furthermore, as previously mentioned, CRHR1 activation has been shown to stimulate several signaling pathways. Consequently, despite the fact the PKA seems to have a major role in this acute stress, CRHR1-mediated, executive dysfunction, it is also necessary to explore the PKC as another potential candidate contributing to these effects. However, the current data constitutes an important and relevant contribution to the neurochemical and molecular mechanisms responsible for the acute stress-induced cognitive dysfunction that strongly impacts our modern society,

and posits CRHR1 as a potentially attractive therapeutic target to ameliorate such deficits. In other words, these results point to the potential use of CRHR1 antagonists as anti-stress drugs, in order to improve quality of life and avoid the detrimental consequences of acute stress, allowing us to successfully cope with challenging scenarios in our everyday life.

VII. REFERENCES References

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VIII. ABBREVIATIONS

AC	Anterior Cingulate
ACSF	Artificial Cerebrospinal Fluid
ACTH	Adenocorticotropic Hormone
ADHD	Attention Deficit Hyperactivity Disorder
AKAP	A-Kinase Anchoring Proteins
ATP	Adenosine Triphosphate
AVP	Arginin Vasopressin
BA	Broadman Area
BLA	Basolateral Amygdala
BNST	Bed Nucleus of the Stria Terminalis
CaMKII	Ca+/calmodulin-dependent protein kinase II
cAMP	cyclic Adenosine Monophosphate
CeA	Central Nucleus of the Amygdala
CoIP	Co-immunoprecipitation
CORT	Corticosterone
CREB	cAMP Response Element Binding Protein
CRF	Corticotropin Releasing Factor
CRH	Corticotropin Releasing Hormone
CRHR1	Corticotropin Releasing Hormone Receptor 1
CRHR2	Corticotropin Releasing Hormone Receptor 2
DLPFC	Dorsolateral Preforntal Cortex
ERK 1/2	Extracellular-signal Regulated Kinase 1/2
GABA	gamma-Amino-Butyric Acid
GC	Glucocorticoids
GEF	Guanyl-nucleotide Exchange Factor
GPCR	G-protein Coupled Receptors
GR	Glucocorticoid Receptors
HPA	Hypothalamus Pituitary Adrenal Axis
IL	Infralimbic
LC	Locus Coeruleus
LTP	Long-Term Potentiation

MAPK	Mitogen Activated Protein Kinase
mPFC	medial Prefrontal Cortex
MR	Mineralcorticoid Receptors
OCD	Obsessive Compulsive Disorder
PFC	Prefrontal Cortex
PKA	Protein Kinase A
PKA-C	Protein Kinase A Catalytic subunit
PKA-R	Protein Kinase A Regulatory subunit
PKB	Protein Kinase B
PKC	Protein Kinase C
PL	Prelimbic
POMC	Proopiomelanocortin
PVN	Paraventricular Nucleus
REM	Rapid-Eye Movement
rIPFC	Right Inferior Prefrontal Cortex
SAM	Sympathetic Adrenal Medullary Axis
UCN	Urocortins
VMPFC	Ventromedial Prefrontal Cortex

IX. TABLE OF FIGURES

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XI. CURRICULUM VITAE

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