PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE

Facultad de Ciencias Biológicas
Programa de Doctorado de Ciencias Biológicas
Mención Biología Celular y Molecular

Modulatory role of the dopamine D1 receptor and Type-2 corticotrophin releasing factor receptor on the basolateral amygdala to prefrontal cortex synapses

Hector Eduardo Yarur Castillo

Director de tesis: Dr. Katia Gysling

FINANCIAMIENTO

Esta tesis recibió los siguientes apoyos de financiamiento:

- Premio otorgado por el programa de doctorado de ciencias biológicas por ser el primero de la generación de Bioquímica en ingresar al programa de doctorado en la Pontificia Universidad Católica de Chile.
- Beca para estudio de doctorado en Chile, CONICYT, años 2012-2016.
- Beca de Extensión para estudios de Doctorado en Chile, CONICYT, año 2016.
- Beca de apoyo financiero (Categoría 1A: Visita del solicitante a otro laboratorio) del Comité de Ayuda y Educación en Neuroquímica (CAEN).
- FONDECYT N° 1150244 para Katia Gysling.

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ABBREVIATIONS

5-HT2: 5-hydroxytryptamine receptor 2

ACTH: adrenocorticotropic hormone

AGm: medial agranular

AC: anterior cingulate

aSvg-30: antisauvagine-30

BDA: biotinylated dextran amine

BLA: basolateral amygdala

BNST: bed nucleus of the stria terminalis

BSA: bovine serum albumin

cmPFC: contralateral mPFC

CRF: corticotropin-releasing factor

CRF1: type-1 CRF receptor

CRF2: type-1 CRF receptor

D1R: dopamine D1 receptor

D2R: dopamine D2 receptor

D3R: dopamine D3 receptor

D4R: dopamine D4 receptor

D5R: dopamine D5 receptor

DR: dorsal raphe nucleus

ERK: extracellular–regulated kinases

EPSC: excitatory postsynaptic currents

EPSP: excitatory postsynaptic potential

GABA: gamma-aminobutyric acid

GPCR: G protein coupled receptor

HEK293T: human endothelial kidney 293t

HFS: high frequency stimulation

HPA: hypothalamic-pituitary-adrenal

IL: infralimbic

Kyn: kynurenic acid

LFP: local field potential

LS: lateral septum

LTP: long term potentiation

MD: midline thalamus

mRNA: messenger ribonucleic acid

mPFC: medial prefrontal cortex

OX1: orexin receptor 1

PFC: prefrontal cortex

PL: prelimbic

PVN: paraventricular nucleus of the hypothalamus

REST: RE1-Silencing Transcription factor

UCNI: Urocortin I

UCNII: Urocortin II

UCNIII: Urocortin III

Vglut1: vesicular glutamate transporter 1

VH: ventral hippocampus

VTA: ventral tegmental area

RESUMEN

Dopamina y el factor liberador de corticotropina (CRF) regulan varios procesos de modulación sináptica. El sistema de CRF está compuesto de cuatro ligandos, CRF, urocortina I, urocortina II y urocortina III; y de dos receptores acoplados a proteína G (GPCR), receptor de CRF tipo-1 (CRF1) y receptor de CRF de tipo-2 (CRF2). La dopamina es capaz de unirse a dos familias de receptores GPCRs, los llamados receptores de dopamina de tipo-1, compuesto por D1R y D5R, y los receptores de dopamina de tipo-2, compuesto por D2R, D3R y D4R. Se ha descrito que D1R tiene un rol regulatorio de la liberación de glutamato y dopamina en el área del tegmento ventral (VTA), en animales previamente expuestos a cocaína. En el caso de CRF2, se ha mostrado que regula la liberación de glutamato y dopamina en el VTA dependiente de la generación de un estímulo estresante sobre los animales que fueron administrados repetidas veces con cocaína. Además, se ha descrito que CRF2 y D1R son capaces de formar un complejo heteromérico que modifica la localización subcelular de CRF2 y D1R, cuando se sobre-expresan en conjunto en cultivos celulares de HEK293t. Sin embargo, no hay evidencia de la modulación que ejercen los agonistas para CRF2 y D1R sobre el heterómero CRF2/D1R y de su posible papel regulatorio en alguna vía que involucre la regulación por CRF y dopamina. Basados en estos antecedentes decidimos estudiar la regulación del heterómero CRF2/D1R por sus ligandos, CRF y dopamina, utilizando una preparación en la cual ambos receptores se sobre-expresaran como la línea celular HEK293t. Nuestros resultados revelaron que el heterómero CRF2/D1R es regulado por dopamina y CRF, tanto a nivel de localización, como en su interacción y señalización. La presencia de CRF2 en la membrana plasmática se reduce al ser co-expresado con D1R y en presencia de los agonistas de CRF y dopamina. La interacción CRF2/D1R aumenta, pero su señalización se reduce solo al co-infundir CRF y dopamina. Por lo tanto, los resultados indican que dopamina y CRF regulan al heterómero CRF2/D1R reduciendo la expresión de CRF2 en la membrana y modificando la señalización de ambos receptores.

Adicionalmente, se ha descrito que en la corteza prefrontal (PFC) se expresa el D1R y modula la actividad que tiene la amígdala basolateral (BLA) hacia la PFC, pero no se ha descrito la expresión de CRF2. Controversialmente, se ha descrito efectos mediados por CRF2 en la PFC. Hasta este trabajo no se ha descrito la expresión de CRF2 en la PFC, lo que nos llevó a analizar donde se expresaba el CRF2 en PFC y si co-localizaba con D1R, modulando la

transmisión sináptica entre BLA y PFC. Nuestros resultados mostraron que CRF2 se expresa en terminales sinápticos en la PFC. Además, el mRNA para CRF2 se encuentra en los terminales sinápticos de la PFC. Los datos muestran que CRF2 co-localiza con D1R en terminales glutamatérgicos provenientes de la BLA hacia la PFC. Además, CRF2 modula negativamente la liberación de glutamato desde la BLA hacia la PFC, siendo esta modulación eliminada cuando se co-infunden antagonistas de ambos receptores en la PFC. La potenciación a largo plazo (LTP) entre la BLA y PFC es modulada por CRF2 y D1R. Por lo tanto, los resultados indican que CRF2 se expresa en la PFC y en conjunto con D1R modulan el glutamato y la plasticidad entre la BLA y PFC.

Los resultados de esta tesis ofrecen nuevos conocimientos sobre la interacción entre el sistema CRFérgico y dopaminérgico en la PFC. Entregan evidencia relevante de la regulación del heterómero CRF2/D1R por sus agonistas CRF y dopamina. Además, documentan la expresión de CRF2 en terminales nerviosos que inervan la PFC con una función regulatoria de CRF2 sobre la transmisión sináptica entre la BLA y la PFC.

ABSTRACT

Dopamine and corticotropin-releasing factor (CRF) regulate several synaptic processes. The CRF system is composed of four ligands: CRF, urocortin I, urocortin II and urocortin III; and two G-protein coupled receptors (GPCR), type-1 CRF receptor (CRF1) and type-2 CRF receptor (CRF2). Dopamine can bind to two families of GPCRs, the type-1 dopamine receptor, composed of D1R and D5R receptors, and the type-2 dopamine receptor, composed of D2R, D3R and D4R receptors. It has been described that D1R has a regulatory role in the release of glutamate and dopamine in the ventral tegmental area (VTA), in animals previously exposed to cocaine. In the case of CRF2, it has been shown that regulates glutamate and dopamine release in the VTA, dependent on the generation of a stressful stimulus on animals that were administered repeatedly with cocaine. Furthermore, it has been described that CRF2 and D1R are capable of forming a heteromeric complex that modify the subcellular localization of CRF2 and D1R when both receptors are overexpressed in HEK293t cells. However, there is no evidence of the modulation exerted by CRF2 and D1R agonists upon the CRF2/D1R heteromer and of the possible role of the heteromer in the regulation of a neural pathway where CRF2 and D1R are present.

Based on this background, we decided to study the regulation of the CRF2/D1R heteromer by its ligands, CRF and dopamine, overexpressing both receptors in the HEK293t cell line. Our results revealed that the CRF2/D1R heteromer is regulated by dopamine and CRF, at the level of localization, interaction and signaling of both CRF2 and D1R. The presence of CRF2 in the plasma membrane was reduced when is co-expressed with D1R and by the presence of both CRF and dopamine agonists. The CRF2/D1R interaction increased, but its signaling was reduced, by the co-infusion of CRF and dopamine. Therefore, the results indicate that dopamine and CRF regulate the CRF2/D1R heteromer by reducing the expression of CRF2 in plasma membrane and by modifying the signaling of both receptors.

It has been described that D1R is expressed in the prefrontal cortex (PFC) where it modulates the neuronal transmission between basolateral amygdala (BLA) and PFC. There is not anatomical evidence of the expression of CRF2 in PFC, even though there has been reported effects mediated by CRF2 in the PFC. Thus, we decided to further analyze whether CRF2 is expressed in PFC and if it co-localizes with D1R, modulating the synaptic transmission

between BLA and PFC. Our results showed that CRF2 are expressed in synaptic terminals of the PFC. In addition, we documented the presence of CRF2 mRNA in synaptic terminals of the PFC. Our results also showed that CRF2 co-localizes with D1R in glutamatergic terminals of the PFC originated in the BLA. In addition, CRF2 negatively modulates the release of glutamate from BLA to PFC. This negative modulation was eliminated when antagonists for both CRF2 and D1R were co-infused in PFC. In addition, we observed that the long-term potentiation (LTP) between BLA and PFC is modulated by CRF2 and D1R. Thus, the results indicate that CRF2 is expressed in the PFC and together with D1R modulate glutamate release and plasticity between BLA and PFC.

The results of this thesis offer new insights into the interaction between the CRFergic and dopaminergic systems in the PFC. They provide relevant evidence of the regulation of the CRF2/D1R heteromer by CRF and dopamine agonists. In addition, they document the expression of CRF2 in PFC nerve terminals originated in the BLA. Thus, our results evidence a regulatory function of CRF2 on the synaptic transmission between BLA and PFC.

INTRODUCTION

1. Modulation of the Prefrontal Cortex activity

The prefrontal cortex (PFC) is the most elaborated region of the mammalian brain (Miller and Cohen, 2001). The PFC is not critical for performing simple and automatic behaviors; however, intelligently, modulates our thoughts, actions and emotions by its varied interconnections with other brain areas (Hoover and Vertes, 2007). The PFC is required for behaviors that need to be processed, such as those behaviors that must be guided by internal state or emotions (Goldman-Rakic, 1995). The PFC is a key area for inhibiting inappropriate action to promoting relevant actions (top-down regulation), allowing us to respond properly to a changing situation (Arnsten, 2009).

The rat PFC was divided into medial, orbital and lateral (Ongür and Price, 2000). The medial PFC (mPFC) has been implicated in several processes such as working memory, decision-making, attention, impulsivity and goal directed behavior (Kolb, 1984; Goldman-Rakic, 1995; Floresco et al., 1997; Fuster, 2000). The mPFC has been divided in medial agranular (AGm), anterior cingulate (AC), prelimbic (PL) and infralimbic (IL) cortex (Hoover and Vertes, 2007). The dorsal region of the mPFC (AGm and AC) has been implicated in motor behavior and the ventral region of the mPFC (IL and PL) has been implicated in diverse processes like emotional, cognitive and impulse behavior (Heidbreder and Groenewegen, 2003). The function of the mPFC is carried out processing the information arriving from different inputs such as the midline thalamus (MD), contralateral mPFC (cmPFC), basolateral amygdala (BLA) and ventral hippocampus (VH) (Hoover and Vertes, 2007).

One area that projects heavily to the ventral region of the mPFC is the BLA (Hoover and Vertes, 2007). The BLA is a brain area that sends crucial information about the emotional salience of sensory stimuli to the mPFC (LeDoux, 2000). The BLA to mPFC pathway provides direct excitatory glutamatergic inputs to pyramidal cells and some glutamatergic inputs to mPFC interneurons (Shinonaga et al., 1994; Gabbott et al., 2006). The BLA exerts two distinct types of responses, an excitatory modulation over the pyramidal cells and a more commonly fast ‘feed-forward’ GABA mediated inhibition over mPFC cells (Floresco and Tse, 2007; Dilgen et al., 2013). Interestingly, it has been described that dopamine receptors modulate the activity of mPFC neurons (Tseng et al., 2004; Floresco and Tse, 2007). The

dopamine receptors are G-coupled protein receptors (GPCR) divided in two families: dopamine D1 -like and dopamine D2-like. The dopamine D1 family is composed of dopamine D1 receptor (D1R) and dopamine D5 receptor (D5R). The dopamine D2 family is composed of dopamine D2 receptor (D2R), dopamine D3 receptor (D3R) and dopamine D4 receptor (D4R). The D1 family is generally coupled to Gs and the D2 family is coupled to Gi (Beaulieu and Gainetdinov, 2011). Early studies in the mPFC revealed that D2R decreased excitatory postsynaptic potential (EPSP) amplitude on the deep layers of the mPFC. This D2R effect is dependent on the local GABAergic activity (Tseng and O´Donnell, 2007). On the other hand, the effect of D1R in the mPFC is to reduce the glutamatergic and GABAergic components in the deep layers of the mPFC (Law-Tho et al., 1994; Yang and Seamans, 1996; Seamans et al., 2001). D1R and D2R are located across the layers of the mPFC in pyramidal neurons and non-pyramidal interneurons; with a significant increased expression of the receptors on the deep layers (Santana and Artigas, 2017). Interestingly, D1R have been described to be expressed in presynaptic terminal compartments of the mPFC (Paspalas and Goldman-Rakic, 2005). The expression of dopamine receptors on the mPFC can explain in part the effects of D1R and D2R in the mPFC. Furthermore, the D1R and D2R have opposite effects on the inhibitory control of the BLA over the mPFC (Floresco and Tse, 2007). D2R attenuates the BLA-evoked inhibition of the mPFC and D1R attenuates the BLA-evoked firing of mPFC neurons (Floresco and Tse, 2007). This evidence gives dopamine a modulatory role over the BLA transmission to the mPFC.

2. Stress response, CRF system and mPFC

Another factor that disturbs mPFC activity is stress (Snyder et al., 2015; Urban et al., 2017). The stress response plays a key role in the adaptation behavior of the animals in response to a threatening stimulus (LeDoux, 2000). The corticotrophin releasing factor (CRF) plays a significant role in the integration of the endocrine and behavioral responses to stress (Vale et al., 1981). The CRF family plays a role in the regulation of the stress response through the hypothalamic-pituitary-adrenal (HPA) axis. The 41-amino acid peptide CRF is secreted from neurons in the paraventricular nucleus of the hypothalamus (PVN) and induces the secretion of ACTH in the anterior pituitary. ACTH enters in the blood stream accessing the adrenal

gland cortex where it stimulates the secretion of glucocorticoids, which start the response of the body to a stressor (Dedic et al., 2017). The action of CRF is not confined to the HPA axis. There is CRF neurons in several brain nuclei regulating neuronal activity (Hauger et al., 2006; Dedic et al., 2017).

CRF is part of what is called the CRF system comprised of CRF and 3 additional neuropeptides: Urocortin I (UCNI), Urocortin II (UCNII) and Urocortin III (UCNIII), a soluble protein which binds CRF with high affinity (CRFBP), and two GPCR receptors: CRF type-1 (CRF1) and CRF type-2 (CRF2) (Dedic et al., 2017). The CRF peptides act through the CRF receptors, differing in their affinity for the receptors (Dedic et al., 2017). CRF1 and CRF2 are class B1 GPCRs and share 70% amino acid identity (Dautzenberg and Hauger, 2002). CRF receptors differ mostly in their N-terminal extracellular domain (40% identity) (Dautzenberg and Hauger, 2002). CRF1 has only one functional splice variant expressed in the brain. In contrast, CRF2 has three functional splice variants in humans (α, β and γ) and two in rodents (α and β). CRF2α is the major splice variant in rodents (Chalmers et al., 1996). CRF receptors share high expression in the olfactory bulb, bed nucleus of the stria terminalis (BNST), lateral septum (LS), PVN and dorsal raphe nucleus (DR), but CRF1 is more highly expressed in the brain than CRF2 (Van Pett et al., 2000). CRF receptors couple primarily to a Gs protein, but it has been described that they can bind and activate other G proteins as well (Grammatopoulos and Chrousos, 2002; Hillhouse and Grammatopoulos, 2006).

Stressful stimuli may produce a malfunction of the activity in the mPFC developing a pathological state (Urban et al., 2017). In the stress response, the amygdala is a crucial brain area regulating the effects of stress on memory formation (McGaugh and Roozendaal, 2002). The interconnectivity between the BLA and mPFC is crucial in the establishment of emotional information to guide behavior (Schoenbaum et al., 2006). Interestingly, the exposure of animals to an acute stressful stimulus blocks the induction of plasticity in the transmission between BLA and mPFC (Maroun and Richter-Levin, 2003). In the mPFC, CRF is expressed in neurons across all layers (Yan et al., 1998). CRF1 is widely expressed in pyramidal cells of the mPFC and has been implicated in the emotional adaptation to stress (Uribe-Mariño et al., 2016). In contrast, there is no conclusive evidence showing the expression of CRF2 in the mPFC (Van Pett et al., 2000). Figure 1 describes the major

anatomical aspects of the CRF system in the mPFC. Guan and collaborators (Guan et al., 2014) described that a CRF2 antagonist in the mPFC reduces conditioning of the animals to a psychostimulant, like cocaine suggesting that CRF2 should be present in some neuronal element of the mPFC.

The evidence that dopamine modulates the transmission between BLA and mPFC (Tseng et al., 2004; Floresco and Tse, 2007) together with the information that stress also modulates this neuronal pathway suggest that there may be an interaction between the mPFC dopamine and CRF system. Furthermore, there is evidence showing an interaction between dopamine and the CRF system modulating the BLA-mPFC synaptic transmission (Orozco-Cabal et al., 2008). Thus, it would be interesting to further study the mechanisms by which dopamine and CRF system modulate BLA-mPFC transmission.

3. GPCR heteromers and synaptic modulation

The GPCRs are the largest family of transmembrane receptors proteins. Evidence shows that GPCRs exist as dimer/oligomer complexes that can be formed by the same molecules (homomers) or by different molecules (heteromers) (Milligan, 2009). The formation of a GPCR heteromer leads to a new functional entity that possesses different biochemical characteristics in comparison to the original monomers composing the heteromer (Terrillon and Bouvier, 2004; Ferre et al., 2007; Franco et al., 2007). The formation of a GPCR heteromer would give additional or different characteristics to the GPCR monomers. For example: the proper functionality of some GPCRs, the pharmacological properties of each individual GPCR, changes in the signal transduction of each GPCR and changes in the endocytosis properties (Terrillon and Bouvier, 2004).

There is diverse evidence showing a synergistic role between the dopamine and CRF systems (Wang et al., 2005; Hahn et al., 2009; Blacktop et al., 2011). Interesting evidence shows the modulatory role of CRF2 with dopamine receptors on glutamatergic synapses in

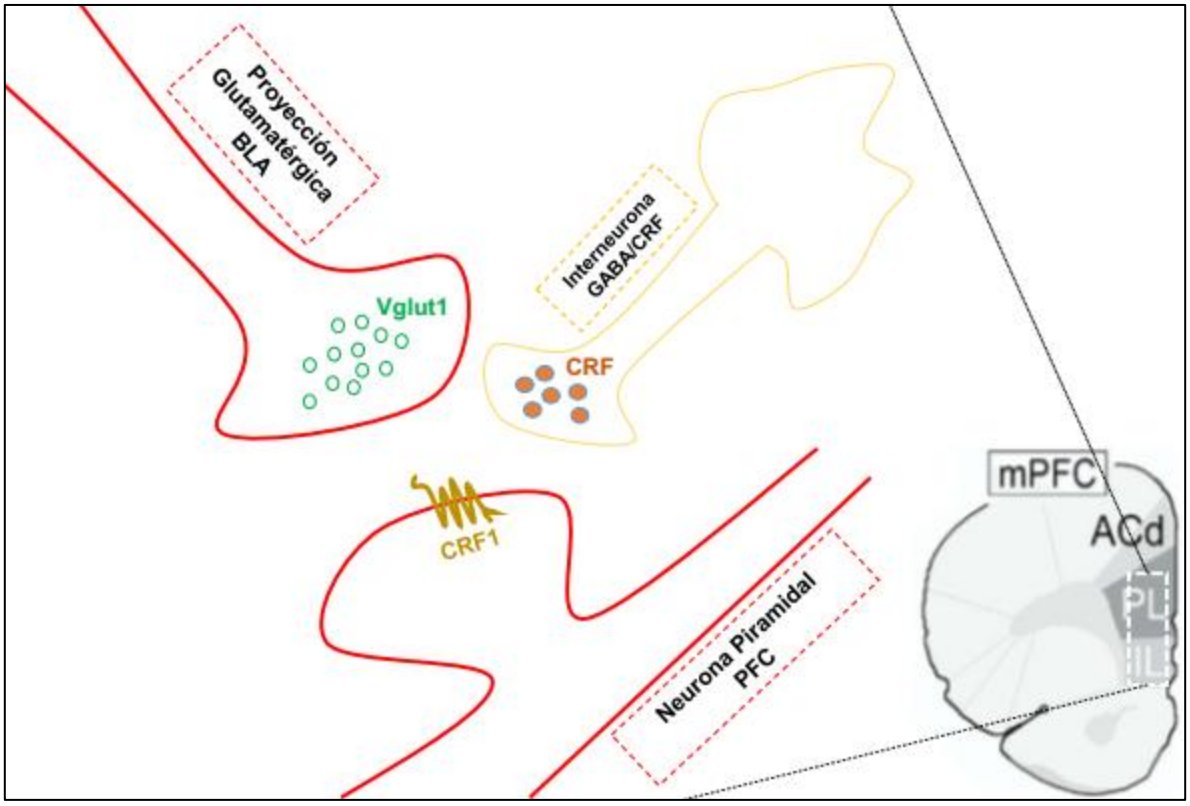

Figure 1. Summary of the known anatomical evidence of the CRF system in the mPFC. The image summarizes the knowing evidence of the CRF system in mPFC. CRF peptide is expressed in mPFC GABAergic interneurons and the CRF1 are expressed in mPFC pyramidal neurons.

different brain areas (Liu et al., 2005; Wang et al., 2007; Williams et al., 2014). In the mPFC, despite the almost undescribed expression of CRF2, it has been described a modulatory effect of CRF2 in the BLA-mPFC synapses, and it is synergically regulated by the activation of D1R (Orozco-Cabal et al., 2008). It has been shown that D1R and CRF2 can form a stable heteromer in HEK293t cell (Fuenzalida et al., 2014). It is tempting to suggest that D1R and CRF2 acting as a heteromer could modulate the transmission between BLA and mPFC.

The above data raises the following questions: Are CRF2 and D1R co-expressed in the BLA-mPFC synapses? Are CRF2 and D1R involved in the neuronal transmission of BLA-mPFC synapses? Would agonist for CRF2/D1R heteromer modulate the intracellular signaling? These questions are addressed in this thesis.

HYPOTHESIS

Dopamine D1 and corticotrophin releasing factor type-2 receptors modulate the glutamatergic synapses between BLA and mPFC.

GENERAL AIM

To elucidate the functional consequences of the heterodimerization of D1R and CRF2 and their eventual role in the glutamatergic synapse between BLA and mPFC.

SPECIFIC AIMS

1. **To evaluate whether the agonists modulate the cellular localization, interaction and signaling of the D1R/CRF2 heteromer.**
   - 1.1. To determine if D1R and CRF agonists modulate the cellular localization of D1R and CRF2.
   - 1.2. To determine if D1R and CRF agonists facilitate or reduce the heteromerization of D1R and CRF2.
   - 1.3. To determine and characterize the signaling of the D1R/CRF2 heteromer.

2. **To evaluate the modulatory role of D1R and CRF2 in the synaptic transmission between the BLA and mPFC.**
   - 2.1. To determine if CRF2 is expressed in the mPFC.
   - 2.2. To determine and characterize mPFC inputs containing D1R and CRF2.
   - 2.3. To determine if BLA projections to the mPFC contain D1R and CRF2.
   - 2.4. To determine if CRF2 and D1R modulate mPFC extracellular glutamate levels induced by the stimulation of BLA.
   - 2.5. To determine the functional consequences of D1R and CRF2 in the BLA-mPFC synapses.

CHAPTER I

PHARMACOLOGICAL PROPERTIES OF THE CORTICOTROPHIN RELEASING FACTOR TYPE-2α RECEPTOR / DOPAMINE D1 RECEPTOR HETEROMER: EVIDENCE OF A CROSSTALK BETWEEN BOTH RECEPTORS

Submitted to Molecular Neurobiology

AUTHORS:

Hector E. Yarur, Maria E. Andres and Katia Gysling

TITLE:

PHARMACOLOGICAL PROPERTIES OF THE CORTICOTROPHIN RELEASING FACTOR TYPE-2A RECEPTOR/DOPAMINE D1 RECEPTOR HETEROMER: EVIDENCE OF A CROSSTALK BETWEEN BOTH RECEPTORS

AUTHORS AFFILIATIONS:

Department of Cellular and Molecular Biology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, 8331150 Santiago, Chile.

CORRESPONDING AUTHORS:

Katia Gysling

Telephone number: +562 2354 2659

Email: kgysling@bio.puc.cl

ABSTRACT

Dopamine and corticotrophin releasing factor (CRF) are neurotransmitters implicated in stress-related addictive behavior. Cocaine treatment enhances glutamatergic transmission in the ventral tegmental area (VTA), dopamine and CRF receptors play a significant role in the modulation that cocaine exerts over VTA glutamatergic transmission. Dopamine D1 receptors (D1R) and CRF type-2α receptors (CRF2α) are able to form a heteromer in cell lines, that confers D1R the ability to mobilize intracellular calcium. The role of dopamine and CRF on the modulation of the CRF2α/D1R heteromer is unknown. In the present study, we evaluated the functional consequences of CRF2α/D1R heteromerization in the signaling and localization of both receptors co-expressed in HEK293T cells. The presence of dopamine in the incubation medium significantly decreased the amount of CRF2α on the cell surface of cells expressing both receptors. We also observed that the presence of both receptor agonists increased the interaction between CRF2α and D1R. Mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) activation by each agonist alone was significantly decreased when the cells were co-incubated with dopamine and CRF. These results provide new evidence of the functional consequences for the heteromerization between CRF2α and D1R. These changes in signaling and localization of CRF2α and D1R when they are heteromerized could be involved in the cocaine enhance of glutamatergic transmission in the VTA.

KEYWORDS:

CRF2, D1R, Heteromer, ERK, Internalization, Interaction.

INTRODUCTION

Drug addiction is a chronic disorder characterized by compulsive drug use despite adverse consequences (Everitt, 2014). One critical component of the neuronal circuits mediating the use of addictive drugs are the midbrain dopamine neurons located in the ventral tegmental area (VTA) (Kauer and Malenka, 2007; Sesack and Grace, 2010). It has been shown that all drugs of abuse increase dopamine levels in the midbrain neuronal circuit output to nucleus accumbens (NAc) (Di Chiara and Imperato, 1988). In addition to dopamine, stress has been implicated in the psychopathology of addiction (Corominas et al., 2010; Gysling, 2012). The exposure to drugs of abuse and/or stressful stimuli induce relapse to drug seeking by a mechanism of strengthening excitatory synapses over VTA dopamine neurons (Saal et al., 2003; McFarland et al., 2004; Kauer, 2004). The neuropeptide corticotrophin releasing factor (CRF) has been implicated in the mechanisms by which stress triggers relapse to drug-seeking behavior (Shaham et al., 1997; Erb et al., 1998). CRF acts through two G-protein-coupled receptors (GPCR), type 1 (CRF1) and type 2 (CRF2) CRF receptors (Grammatopoulos and Chrousos, 2002). Both CRF receptors have been involved in the mechanisms determining stress-induced relapse to drug seeking behavior (Wang et al., 2005; Hahn et al., 2009; Blacktop et al., 2011). Interestingly, addictive drugs induce plastic changes that affect CRF2 signaling (Liu et al., 2005; Wang et al., 2007; Orozco-Cabal et al., 2008; Williams et al., 2014). It has been shown that exposure to a stressful stimulus induces VTA glutamate release only in animals that has been repeatedly exposed to cocaine before (Wang et al., 2005). This sensitization of VTA glutamate in cocaine-exposed rats is mediated by CRF/CRF2 (Wang et al., 2007). On the other hand, it has been shown that glutamate levels in the VTA are modulated by the dopamine D1 receptor (D1R) (Kalivas and Duffy, 1995). Furthermore, intra-VTA infusion of a D1R antagonist prevented glutamate release induced

by cocaine in animals repeatedly exposed to cocaine (Kalivas and Duffy, 1998). These data suggest a functional interaction between CRF2 and D1R to modulate stress-induced release of VTA glutamate in animals repeatedly exposed to cocaine. Recently, it has been described that CRF2 and D1R can form a heteromeric complex and the D1R in the presence of CRF2 change his signaling pathway to increase the intracellular calcium by a D1R agonist (Fuenzalida et al., 2014). Thus, the heteromer formed by CRF2/D1R could be implicated in CRF-induced glutamate release observed in animals exposed to repeated cocaine treatment.

It has been described that heteromerization of GPCRs could change the cellular localization of one or both receptors involved in the interaction (Terrillon and Bouvier, 2004; Magalhaes et al, 2012; Milan-Lobo et al, 2013). In addition, the heteromerization could modulate some properties of the GPCRs, like signaling and desensitization (Magalhaes et al., 2012; Slater et al., 2016). Interestingly, it has been shown that CRF1 enhances the signaling of the serotonin receptor (5-HT2) by the recycling of CRF1, increasing the amount of the 5-HT2 in the plasma membrane (Magalhaes et al., 2010). Thus, the recycling of one of the GPCRs of a heteromer can modulate its partner GPCR properties, such as signaling and cellular localization. D1R is capable of rapid endocite in the present of an agonist and this endocitosys is necessary for D1R signaling (Kotowski et al., 2011). On the other hand, CRF2 is predominantly located intracellularly (Tu et al., 2007; Waselus et al., 2009). Considering the previous information, we studied the impact of the heteromerization of D1R and CRF2 in their response to their agonists. To address this question, we used heterologous expression of D1R and CRF2 in HEK293T cells and determined how D1R and CRF2 agonists modulate the properties of the D1R/CRF2 heteromer complex. We provide evidence that D1R and CRF2 agonists modulate the localization, interaction and signaling of the D1R/CRF2 heteromer.

Material and Methods

Cell culture and transfection

HEK293T cells were grown with DMEM (Gibco) supplemented with 10% FBS (HyClone Labs), 1% penicillin/streptomycin 100× (Gibco), and 2 mM GlutaMax (Gibco). Plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and as previously described (Fuenzalida et al 2014). Experiments were performed 48 hrs after transfecting the plasmids.

Expression vectors

Plasmids pcDNA3.1/myc-His/D1R, pcDNA3.1/CRF_2^{flag}, pcDNA3.1/D1R and pcDNA3.1/CRF_2 were previously obtained in our laboratory (Fuenzalida et al 2014).

Pharmacological assays

For plasma membrane expression assays, HEK293T cells, 48 hrs post-transiently transfected, were serum-starved for 2 hrs in DMEM free-serum before drug treatments. Cells were incubated for 5 min with: human/rat CRF (H-2435; Bachem) plus/or DA (H-8502; Sigma) at the concentrations indicated. After incubation, cells were prepared for plasma membrane protein biotinylation or for protein extraction.

To measure the amount of phosphorylated extracellular signal-regulated kinase (ERK1/2), post-transfected cells were serum-starved for 2 hrs in DMEM free-serum before drug treatments. Cells were incubated with: human/rat CRF (H-2435; Bachem) plus/or DA (H-8502; Sigma), antisauvagine-30 (300 nM, Tocris), SCH23390 (1 µM, Tocris) at the concentrations and times indicated. After incubation, cells were prepared for protein extraction with a lysis buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 1mM EDTA, 0.1%

SDS and 1% triton X-100) with the protease inhibitor NaF (10 mM; Merck) and the phosphatase inhibitor Na_3O_4V (2 mM; Sigma).

Biotinylation of plasma membrane proteins

Transiently transfected HEK293T cells (8×10^6), exposed to CRF and/or DA were washed three times with cold PBS (pH 8.0) and incubated for 30 min with EZ-Link™ Sulfo-NHS-Biotin (0.5 mg/mL, Thermo Scientific) in PBS (pH 8.0). Then, the cells were washed three times with cold glycine (100 mM) in PBS (pH 7.4) and one time with PBS (pH 7.4). The cells were detached from the plate in PBS for protein extraction.

The precipitation of biotinylated protein was made following the manufacture instructions for NeutrAvidin® Agarose Resin (Thermo Scientific). Three hundred and fifty µg of biotinylated supernatant proteins were incubated with 80 µL NeutrAvidin® Agarose in a rotator at 4°C for 2 hrs. The homogenates were centrifuged at 1750 rpm to collect the NeutrAvidin® beads and washed two times with lysis buffer and two more times with cold PBS. Finally, the precipitated biotinylated proteins were incubated with loading buffer 2× and heated at 37°C for 1 hr to perform a western blotting.

Protein extraction and immunoprecipitation

After treatments, HEK293T cells were collected in ice-cold PBS (pH 7.4) and washed with PBS. Then, the cells were centrifuged at 1500 rpm for 5 min and resuspended in lysis buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 1mM EDTA, 0.1% SDS and 1% triton X-100) with protease inhibitors cOmplete Mini (Roche) as described by Rutz et al. (2006), and homogenized with a piston sonicator (Cell Ultrasonic Disrupter) with three pulses of 10s and then incubated for 1 hr in a rotator at 4°C. Finally, the homogenate was centrifuged at 15000 rpm for 15 min at 4°C. The supernatant was collected, and the protein concentration

determined with the Micro BCA™ Protein Assay Kit (Thermo Scientific). Immunoprecipitation was performed as previously described (Fuenzalida et al 2014). Then, 700 µg of the supernatant protein were pre-cleared with TrueBlot® anti-Rabbit Ig IP Agarose Beads (Rockland) and incubated with 1 µg of rabbit anti-myc antibody (Ab9106, Abcam). Loading buffer 2× (8 M urea, 2% SDS, 100 mM DTT, 375 mM Tris, pH 6.8) was added to each sample and heated at 37°C for 1 hr to perform western blotting.

Western blotting

Proteins from HEK293T cells were resolved by 10% SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore). The membranes were incubated overnight at 4°C with primary antibodies and the peroxidase-conjugated secondary antibodies were incubated for 2 hours (1/4000, Jackson ImmunoResearch Laboratories). The primary antibodies used were: mouse anti-FLAG (1:1000, Anti-DYKDDDDK; Clontech); rabbit anti-D1R (1:500, SC-14001; Santa Cruz Biotechnology); rabbit anti-p44/42 ERK1/2; 1:1000, #9102; Cell Signaling Technology); rabbit anti-Phospho-p44/42 (ERK1/2; Thr202/Tyr204) (1:1000, #4370; Cell Signaling Technology); mouse anti-β-Actin (1:3000, SC-47778; Santa Cruz Biotechnology). The membranes were revealed using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology).

Immunofluorescence and confocal microscopy

HEK293T cells were seeded at 7×10^6 cells per well on a 24-well plate on coverslips coated with poly-L-lysine (Sigma). After 48 hrs post transfection, cells were incubated with WGA^{647} (1:1000; Invitrogen) in PBS (pH 7.4), washed two times with PBS and fixed with 4% paraformaldehyde (PFA). Immunofluorescence was done as previously described (Fuenzalida et al 2014). Cells were incubated with the primary antibody rabbit anti-D1R

receptor (1:500; sc-14001, Santa Cruz Biotechnology), overnight at 4ºC in a wet chamber. Then, cells were washed and incubated for 2 hrs with the secondary antibody donkey anti-rabbit AlexaFluor^{Cy3} at room temperature. Cells were washed and mounted with mounting media (Dako).

Image analysis was done as previously described (Fuenzalida et al 2014). Capture of fluorescent images was done with a confocal microscope (Fluoview 1000, Olympus) and Fluoview v6.0 software. Images were digitally obtained with a 100× objective (N.A. 1.4 oil). The stacking of images was done with a Z step of 200 nm per cell. Images were processes using ImageJ software (rsb.info.nih.gov/ij) and deconvolved using the "Iterative Deconvolve 3D" plugin within ImageJ. Manders coefficient was used to analyze colocalization (Manders et al 1993).

Statistical analyses

The statistical analysis was performed with the statistical software GraphPad Prism 6 (GraphPad Software). Data are expressed as the mean ± SEM.

RESULTS

It has been shown that the stimulation of D1R is crucial for regulating the amount of D1R in the plasma membrane and its signaling (Kotowski et al, 2011). To evaluate the stimulation of D1R in our HEK293T cell cultures, we transiently transfected the cells with a construct bearing, human D1R. As shown in Figure 1, D1R colocalized mainly with the plasma membrane marker WGA^{647}. Five min after incubating the cells with 10µM dopamine, D1R immunofluorescence in the plasma membrane significantly increased (Fig. 1A, B).

Next, we performed biotinylation of plasma membrane proteins after incubating the cells with 10µM dopamine, to quantify the change of D1R on the plasma membrane induced by the dopamine treatment (Fig. 1C). Dopamine treatment increased the amount of D1R on the plasma membrane (Fig. 1D), as described by von Zastrow´s group (Vargas and von Zastrow, 2004; Kotowski et al., 2011).

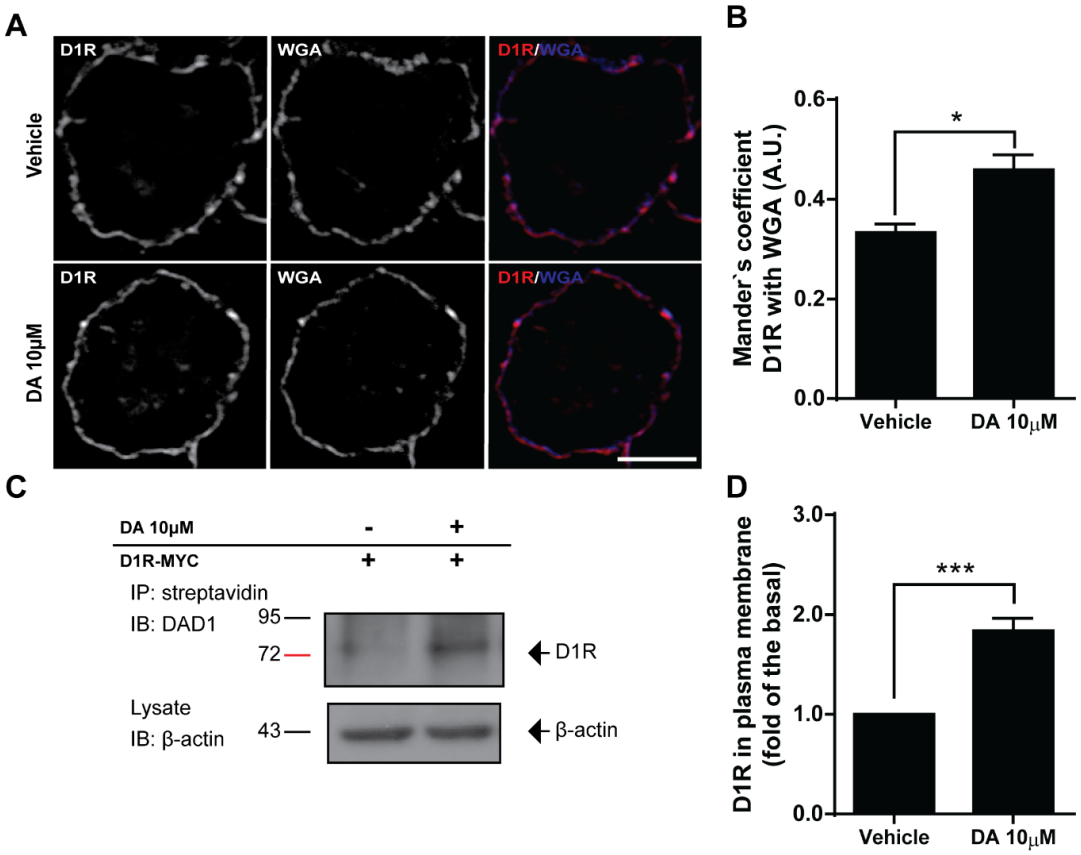

Figure 1. The dopamine treatment increased the D1R on the plasma membrane. (A) Representative 100X confocal image of D1R distribution in HEK293T cells after 5 min incubation with (bottom) or without (top) of 10 μM dopamine. The total pools of D1R were labeled with AlexaFluor^{Cy3}-conjugated secondary antibody (red) and plasma membrane was labeled with WGA^{647} (blue) (scale bar: 10μm). (B) Quantification of co-localization of D1R with WGA after the dopamine treatment. Unpaired Mann-Whitney U test compared to vehicle (*$p<0.05$). Values are expressed as mean ± SEM. (C) Representative western blot of D1R in the plasma membrane after 5 min incubation with (+) or without (-) of 10μM dopamine. The amount of dopamine receptor was normalized by the expression of β-actin. (D) Quantification of dopamine receptor on the plasma membrane after the dopamine

treatment. Unpaired Mann-Whitney U test compared to vehicle (***$p<0.0005$). Values are expressed as mean ± SEM.

Thus, we used the biotinylation assay to evaluate the presence of CRF2α in the plasma membrane after incubating cells expressing CRF2α and D1R with dopamine and/or CRF. Interestingly, the incubation with dopamine decreased the amount of the CRF2α in the plasma membrane of cells that co-expressed CRF2α with D1R (Fig. 2A, B). Furthermore, incubation of HEK293T cells co-expressing CRF2α/D1R with both dopamine (10μM) and CRF (500nM; Magalhaes et al, 2010) produced similar decrease of CRF2 in the plasma membrane than that induced by incubation with dopamine alone. Thus, the stimulation of D1R modulates the amount of CRF2α in the plasma membrane by decreasing the amount of CRF2 in the plasma membrane on cells expressing CRF2α and D1R that are exposed to dopamine.

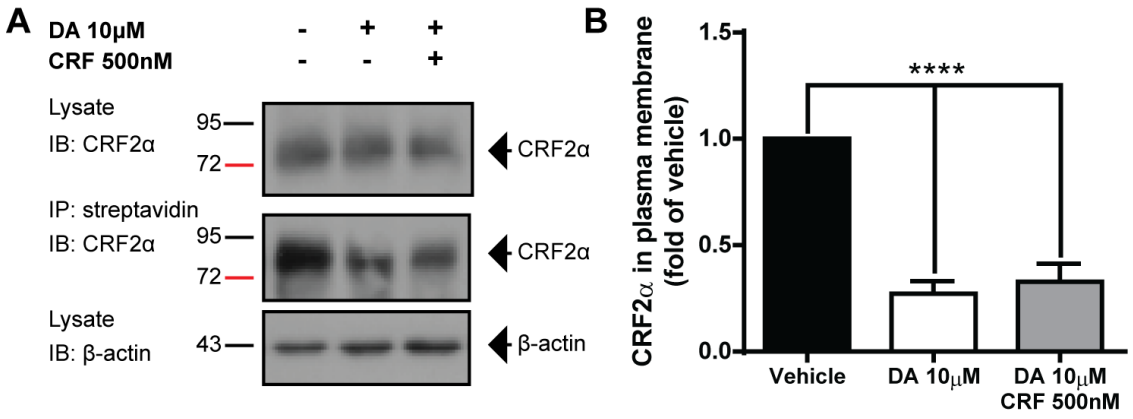

Figure 2. Agonist treatments reduce the plasma membrane expression of CRF2α when is co-expressed with D1R. (A) Representative western blot of CRF2α in the plasma membrane after 5 min incubation of dopamine (10μM) and/or CRF (500nM) of HEK293T cell transiently transfected with CRF2α and D1R. The amount of CRF receptor was normalized by the expression of β-actin. (B) Quantification of CRF2α on the plasma membrane after the agonist treatment in the presence of D1R. One-way ANOVA followed by Bonferroni's multiple comparison post hoc tests compared to CRF2α and CRF2α/D1R respectively (**p<0.005, *** p<0.0005, ****p<0.00005). Values are expressed as mean ± SEM.

For some GPCRs, the stability of the interaction with other proteins is modulated by the presence of their agonists (Magalhaes et al, 2012; O'Dowd et al, 2011; Slater et al 2016). Thus, we tested whether the interaction between CRF2α and D1R depends on the presence of dopamine (Fig. 3A, B). Incubation of cells in the presence of dopamine did not change the amount of CRF2α interacting with D1R in HEK293T cells co-expressing both receptors (Fig. 3A, B). Considering the lack of effect of the dopamine treatment on CRF2α/D1R interaction; we evaluated if the co-treatment with dopamine and CRF could modify the interaction between CRF2α and D1R. Interestingly, when HEK293T cells co-expressing CRF2α and D1R were treated with dopamine (10 μM) and CRF (500 nM), the amount of CRF2α interacting with D1R significantly increased compared to the control (Fig. 3A, B). Thus, the interaction between CRF2α and D1R increased only in the presence of both dopamine and CRF.

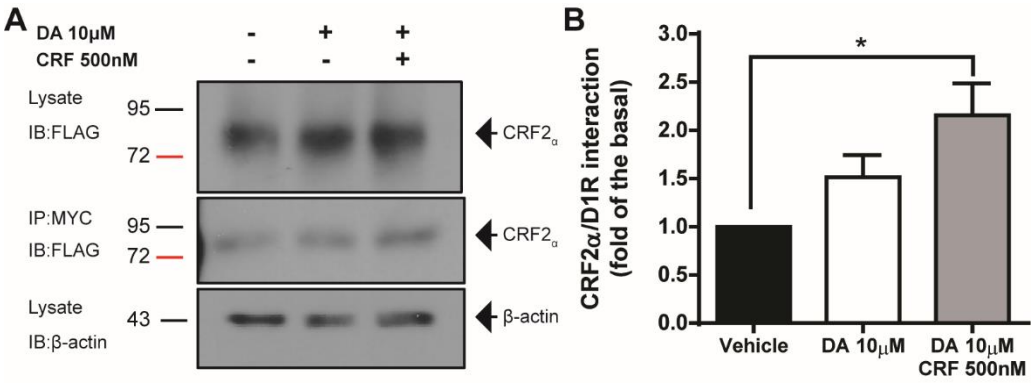

Figure 3. The co-treatment of dopamine and CRF increased the CRF2α/D1R interaction. (A) Representative western blot of the co-immunoprecipitation of CRF2α and D1R in HEK293T cells. The cells were incubated for 5 min with (+) or without (-) dopamine 10μM and/or CRF 500nM. The amount of immunoprecipitated CRF2α was normalized by the expression of CRF2α and β-actin. (B) Quantification of CRF2α/D1R interaction after the dopamine and/or CRF incubation of transiently transfected HEK293T cells. One-way ANOVA followed by Bonferroni's multiple comparison post hoc tests compared to vehicle (*$p<0.05$). Values are expressed as mean ± SEM.

In view of the agonist effects over the heteromer CRF2α/D1R, we evaluated the effects of the agonists in the signaling pathways described for these receptors (Neve et al, 2004; Rossant et al, 1999). To evaluate if the agonists for CRF2α and D1R could modulate the signaling through ERK1/2 we set up the conditions for each receptor agonists to modulate the phosphorylation of ERK1/2. First, we analyzed the CRF concentration-dependent induction of ERK1/2 phosphorylation in HEK293T cells expressing CRF2α. The phosphorylation of ERK 1/2 was dependent on the concentration of CRF in the incubation medium being significantly higher at 500 nM CRF (Fig. 4A). Next, we analyzed the time course of CRF-induced ERK 1/2 phosphorylation after the incubation with 500 nM of CRF. ERK 1/2 phosphorylation induced by CRF 500 nM increased with a peak at 5 min of the incubation of HEK293T cells expressing CRF2α (Fig. 4B). For the D1R, the dopamine concentration-dependent induction of ERK 1/2 phosphorylation was significantly higher at 10 μM dopamine (Fig. 4C). ERK 1/2 phosphorylation induced by 10 μM dopamine significantly increased at 5 min of incubation of HEK293T cells expressing the D1R (Fig. 4D).

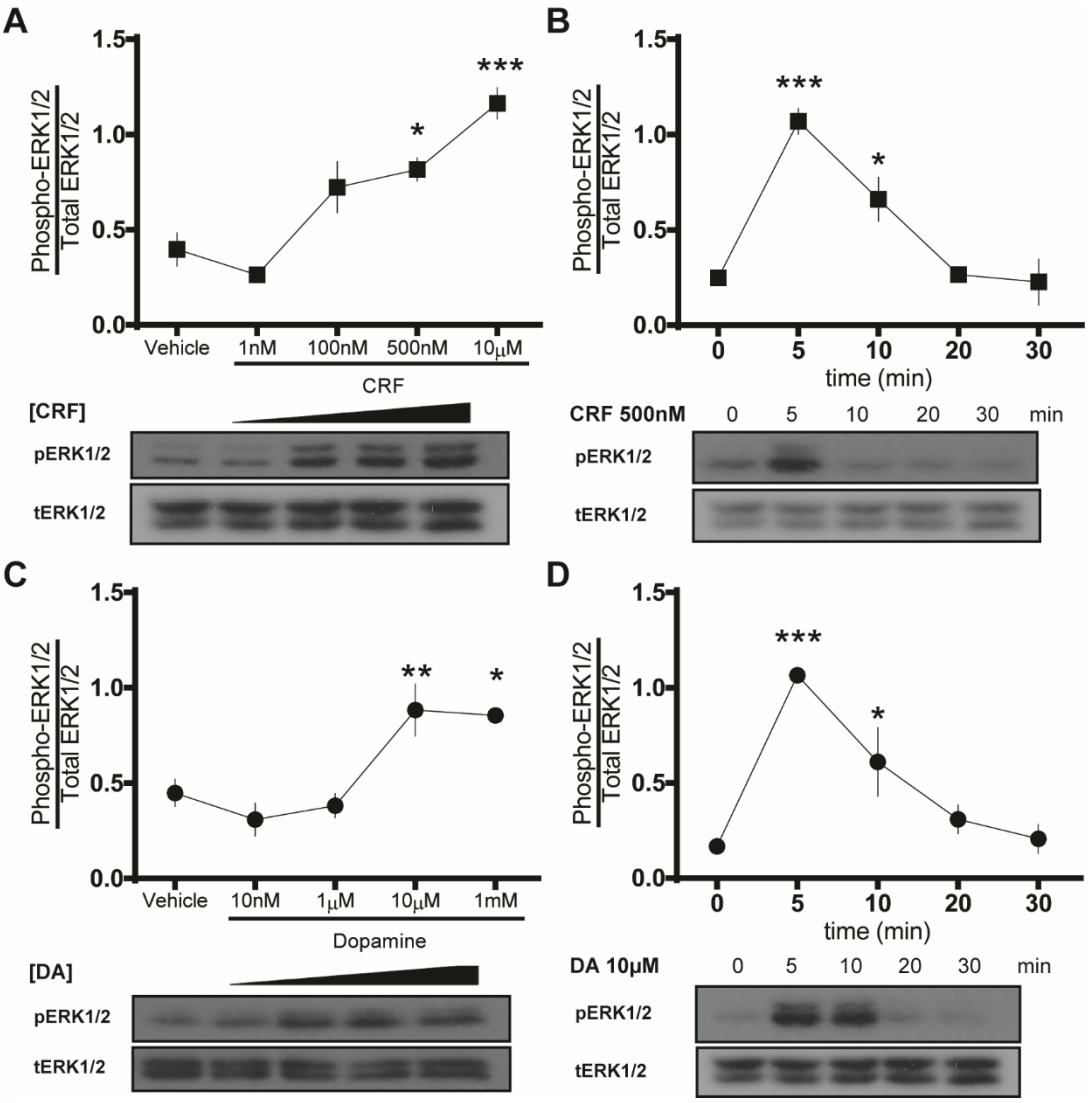

Figure 4. Modulation of the ERK signaling of D1R and CRF2α by dopamine and CRF incubation. (A) Quantification of the phospho-ERK expression in HEK293T cells transiently transfected with CRF2α after 10 min incubation of different concentration of CRF. (B) Quantification of the phospho-ERK expression in HEK293T cells transiently transfected with CRF2α after the incubation of CRF (500nM) at different time points. (C) Quantification of the phospho-ERK expression in HEK293T cells transiently transfected with D1R after 10 min incubation of different concentration of dopamine. (D) Quantification of the phospho-ERK expression in HEK293T cells transiently transfected with D1R after the

incubation of dopamine (10μM) at different time points. The amount of phospho-ERK was normalized by the expression of total ERK. One-way ANOVA followed by Bonferroni's multiple comparison post hoc tests compared to vehicle (*$p<0.05$, **$p<0.005$, *** $p<0.0005$). Values are expressed as mean ± SEM.

Considering the conditions standardized above, we evaluated the phosphorylation of ERK 1/2 in HEK293T cells that expressed the heteromer CRF2α/D1R. The incubation of HEK293T cells expressing the CRF2α/D1R heteromer with dopamine or CRF significantly increased ERK 1/2 phosphorylation (Fig. 5A, C). Interestingly, no-significant changes in ERK 1/2 phosphorylation were observed after incubating the cells with the agonists for both receptors (Fig 5 B, C). The cross-talk in the signaling between CRF2α and D1R in the presence of dopamine and CRF was further evaluated using antagonists for both receptors. The increased in ERK1/2 phosphorylation was lost when dopamine or CRF were incubated in the presence of the partner antagonist antisauvagine-30 (aSvg30, a CRF2α antagonist) or SCH23390 (SCH, a D1R antagonist) (Fig. 5B, C). Overall, the results suggest that the CRF2α/D1R heteromer could have a change in the signaling pathway of the receptors alone, when CRF2 and D1R form a heteromer.

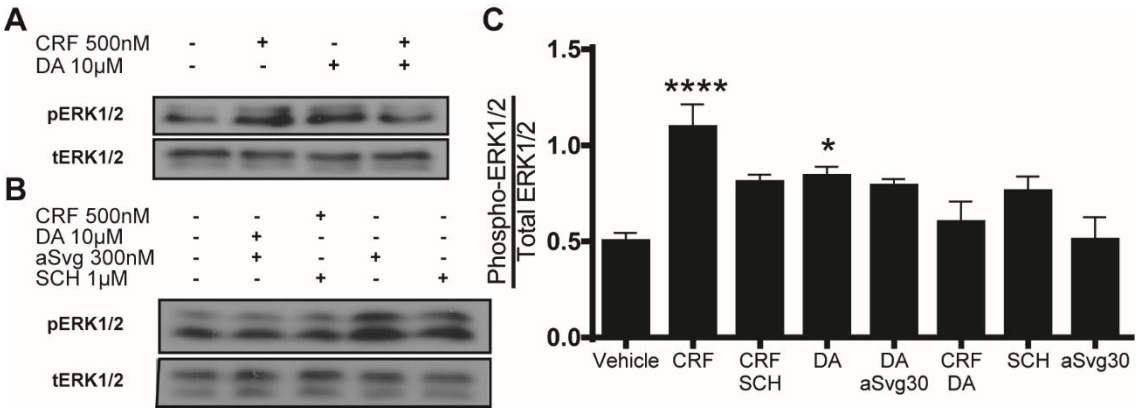

Figure 5. Modulatory effect by dopamine and CRF treatment on the ERK1/2 pathway of the CRF2/D1R heteromer. (A) Representative western blot of phospho-ERK expression in HEK293T cells transiently transfected with CRF2α and D1R. The cells were incubated for 5 min with dopamine (10μM) and/or CRF (500nM). (B) Representative western blot of phospho-ERK expression in HEK293T cells transiently transfected with CRF2α and D1R. The cells were incubated for 5 min with dopamine (10μM), CRF (500nM), SCH23390 (1μM, a D1R antagonist) and/or aSvg (300nM, a CRF2α antagonist). (C) Quantification of the amount of phospho-ERK in A and B, pERK was normalized by the expression of total ERK. One-way ANOVA followed by Bonferroni's multiple comparison post hoc tests compared to vehicle (*$p<0.05$, ***$p<0.0005$). Values are expressed as mean ± SEM.

DISCUSSION

In the present study, we show that the agonists for CRF2α and D1R are capable of modulating CRF2α/D1R heteromer´s properties, modifying the subcellular localization, the interaction and the signaling of the CRF2α/D1R heteromer. Using transient heterologous expression of CRF2α and D1R in HEK293T cells, we have further characterized the CRF2α/D1R heteromer described by Fuenzalida et al. (2014). Interestingly, the presence of dopamine and/or CRF in the incubation medium decreased the amount of CRF2α in the cell surface of HEK293T cells co-expressing CRF2α and D1R. In addition, we observed that the stimulation of D1R does not change the interaction between D1R and CRF2α, but the interaction of CRF2α/D1R increased by the co-infusion of dopamine and CRF. We demonstrate that the stimulation of the D1R reduces the cell surface expression of CRF2α but does not modify the interaction of the CRF2α/D1R heteromer. Our results indicated that the treatment with dopamine plus CRF increases the interaction between D1R and CRF2α. Interestingly, the activation of either D1R or CRF2α increases the amount of phosphorylated ERK1/2, but the co-expression of CRF2α with D1R does not increase the phosphorylated level of ERK1/2 when dopamine and CRF were co-infused.

Our results show that transfected D1R, in the presence of dopamine, increases its amount in the cell surface; similar to what was described by Vargas and von Zastrow (2004). Incubation of HEK293T cells in the presence of dopamine reduced the amount of CRF2α in the cell surface of HEK293T cells co-expressing CRF2α and D1R (Fig. 2). This means that the stimulation of D1R is not capable of increasing the amount of its CRF2α partner in the cell surface. Furthermore, the stimulation of D1R reduced the amount of CRF2α in the cells surface (Fig. 2B). These results suggest that D1R internalized CRF2α by the action of

dopamine and that CRF2α remains intracellularly after the incubation with dopamine. Some GPCRs, in the presence of their agonists, form a stable complex with other proteins (like β-arrestin) impeding receptor resensitization (Oakley et al, 2001; Thomsen et al, 2016). The internalization of D1R with CRF2α in the presence of dopamine tells us about the stability of the CRF2α/D1R heteromer (O'Dowd, 2011). It has been shown that the interaction between D1R and CRF2α is stable enough to change the cellular localization site of CRF2α by using a nuclear localization signal on the D1R (Fuenzalida et al, 2014). We also showed that the interaction between CRF2α and D1R does not depend on the stimulation of D1R by dopamine (Fig. 3). Thus, this finding suggests that the amount of CRF2α interacting with D1R on the cell surface is the same quanta of CRF2α that was internalized with the D1R by the dopamine treatment. Interestingly, the incubation with dopamine and CRF significantly increased the interaction between CRF2α and D1R. This finding could be explained by the amount of CRF2α internalized by CRF (Markovic et al, 2008; Hauger et al, 2013), however the amount of CRF2α internalized by dopamine, compared to the amount internalized by the cotreatment with CRF plus dopamine was similar (Fig. 2). It is tempting to suggest that the CRF2α internalized by the co-treatment of dopamine and CRF, could be the CRF2α that is interacting with D1R intracellularly and that the CRF2α located in the plasma membrane is a homomer. D1R and CRF2α expressed in HEK293T cells colocalized with early-endosome antigen 1 (EEA1, marker of endosomal compartments) without any treatment (data not shown). Ours findings suggest that dopamine plus CRF reduce the CRF2α in the cell surface when is co-expressed with D1R and increase the interaction of CRF2α with the D1R.

Finally, we demonstrated that the peptide CRF activates ERK 1/2 in cells transiently expressing CRF2α. In contrast to some publication were CRF does not active ERK 1/2 by

CRF2α (Brar et al, 2004), but it differs because they used the splice variant CRF2β. Interestingly, the activation ERK 1/2-time curve for CRF2 has an early increase by CRF (Fig. 4A, B). This early activation of ERK1/2 has been described for CRF2α and CRF2β using other agonists for CRF2 such as sauvagine and urocortin II (UCN-II) (Rossant et al, 1999; Brar et al, 2004; Markovic et al, 2008). As already described, D1R could activate ERK signaling (Calabresi et al, 2014). We showed that dopamine can activate ERK1/2 phosphorylation in cells transiently expressing D1R (Fig. 4C, D). It has been shown that the increase in the activation of ERK 1/2 occurred in early phases of the agonist incubation (Navarro et al, 2010; Fiorentini et al, 2011). Dopamine and CRF increased significantly ERK 1/2 activation in cells expressing D1R and CRF2α (Fig. 5). Both CRF-activation and dopamine-increased ERK 1/2 phosphorylation were counteracted by CRF2α or D1R antagonists (Fig. 5A, C). The modulation of an antagonist of one of the receptors in the heteromer to block signals originated by the stimulation of the other receptor is a characteristic described for other heteromers (Ferrada et al, 2009; Navarro et al, 2010; Navarro et al, 2015). Furthermore, we also found mutual agonistic interaction between D1R and CRF2α on ERK 1/2 activation in transfected cells with D1R and CRF2α. The CRF2α agonist increased ERK 1/2 phosphorylation was counteracted when the agonist of D1R was co-incubated in HEK293T cells expressing D1R and CRF2α (Fig. 5A, C). It has been described “cross-talk” in the signaling pathways of some GPCR heteromers, like we described here for the CRF2α/D1R heteromer (Navarro et al, 2010; Navarro et al, 2015). In the case for many GPCR, the β-arrestins are versatile adapters that can form complex with GPCR. β-arrestins recruitment can also trigger activation of alternative signaling cascades, like ERK 1/2 signaling (Luttrell and Lefkowitz 2002). It has been shown that the reduction of β-arrestin 2 blocked ERK 1/2 activation but does not affect other signaling, like Gq-

dependent signaling (Stoppel et al, 2017). For CRF receptors, β-arrestins contribute to the activation of ERK1/2 in response to CRF or UCNs (Bonfiglio et al, 2013; Inda et al, 2016; Markovic et al, 2008). Further studies should address whether the change in ERK 1/2 phosphorylation induced by the co-activation of D1R and CRF2α due to a change in the interaction with some of β-arrestin (Stoppel et al, 2017) or to changes in the downstream signaling of the receptors when their form the CRF2α/D1R heteromer complex (Chun et al, 2013; Fuenzalida et al, 2014). Upon to the activation of ERK 1/2, has been described expression of phosphorylated ERK1/2 on dendrites (Trentani et al, 2002). Furthermore, the activation of ERK1/2 modulates the synaptic plasticity or the intracellular calcium release (Gallagher et al, 2004; Zanassi et al, 2001). Future works needs to focus on defining how the signaling modulation of the CRF2α/D1R heteromer would have implication on the glutamate release or in the synaptic modulation.

In summary, we showed that dopamine could internalize CRF2α when was co-expressed with the D1R but the stimulation of the D1R does not increase the amount of CRF2α in the cell surface. The co-activation of D1R and CRF2α changed the ERK1/2 signaling of each receptor alone. Altogether, the present study demonstrates a functional and pharmacological role of dopamine and CRF in the modulation of the CRF2α/D1R heteromer. The modulation of CRF2α by the stimulation of D1R described here, could explain the release of glutamate induced by CRF2α only in cocaine-experienced animals in which CRF induces the release of glutamate and dopamine (Wang et al, 2005; 2007). The reported findings open new possibilities of the CRF2α/D1R heteromer involvement in cellular localization and specific signaling pathways mediating responses to stress-related relapse to drug seeking behavior.

Funding: This study was funded by FONDECYT grant N° 1150244. H.E.Y was recipient of a PhD fellowship from CONICYT.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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CHAPTER II

INHIBITORY ROLE OF CORTICOTROPHIN RELEASING FACTOR RECEPTOR TYPE-2 IN THE BASOLATERAL AMIGDALAR-MEDIAL PREFRONTAL CORTEX TRANSMISSION OF THE RAT

Submitted to Proceedings of the National Academy of Sciences

CLASSIFICATION:

BIOLOGICAL SCIENCES

TITLE:

INHIBITORY ROLE OF CORTICOTROPHIN RELEASING FACTOR RECEPTOR TYPE-2 IN THE BASOLATERAL AMIGDALAR-MEDIAL PREFRONTAL CORTEX TRANSMISSION OF THE RAT

SHORT TITLE:

REGULATION OF BLA-mPFC TRANSMISSION BY CRF_2

Hector E. Yarur[1], Ignacio Vega-Quiroga[1], Marcela P. Gonzalez[1], Verónica Noches[1] Daniel R. Thomases[3], María E. Andrés[1], Francisco Ciruela[2], Kuei Y. Tseng[3*], Katia Gysling[1*]

AUTHORS AFFILIATIONS:

[1]Department of Cellular and Molecular Biology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, 8331150 Santiago, Chile

[2]Unitat de Farmacologia, Departament Patologia i Terapèutica Experimental, Facultat de Medicina, IDIBELL, Universitat de Barcelona, L'Hospitalet de Llobregat, Barcelona, Spain.

[3]Department of Anatomy and Cell Biology. University of Illinois, Chicago, USA

***CORRESPONDING AUTHORS:**

Katia Gysling
Department of Cellular and Molecular Biology
Faculty of Biological Sciences
Pontificia Universidad Católica de Chile, 8331150 Santiago, Chile
Email: kgysling@bio.puc.cl

Kuei Y Tseng
Department of Anatomy and Cell Biology
College of Medicine
University of Illinois, Chicago, USA

KEYWORDS:

CRF_2, medial prefrontal cortex, basolateral amygdala, Glutamatergic terminals,

ABSTRACT

Basolateral amygdala (BLA) excitatory projections to the medial prefrontal cortex (mPFC) play a key role within the circuitry controlling stress behaviors, pain and fear. Stressful events block synaptic plasticity between BLA and mPFC. Interestingly, in stress responses, corticotrophin releasing factor (CRF) is a key component acting through type 1 and type 2 CRF receptors (CRF_1 and CRF_2). Importantly, while CRF_1 has been involved in mPFC functioning, the participation of CRF_2 in BLA-mPFC synaptic transmission still is unclear. Here, we provide direct evidence of the presynaptic expression of CRF_2 protein and mRNA in mPFC nerve terminals originated in BLA. We also show that dopamine D1 receptors (D1R) colocalize with CRF_2 in these mPFC nerve terminals. Intra mPFC infusion of antisauvagine-30 (aSvg-30), a CRF_2 antagonist, increased mPFC long-term potentiation (LTP) and glutamate levels induced by BLA activation. Interestingly, the increase in LTP and glutamate release observed in the presence of aSvg-30 was significantly inhibited in the presence of SCH23390, a D1R antagonist. These findings show that CRF_2 is expressed in BLA nerve terminals in mPFC and that it exerts an inhibitory role in the synaptic transmission between BLA and mPFC. In addition, our results also show that inhibition of mPFC CRF_2 unmasks the effect of D1R over synaptic plasticity of the BLA to mPFC pathway. Overall, CRF_2 emerges as a new modulator of the BLA-mPFC connection that it has been shown to play a critical role in emotional disorders.

SIGNIFICANCE STATEMENT

The basolateral amygdala (BLA) connects with the medial prefrontal cortex (mPFC) and process emotional information, such as stress response. The action of corticotrophin releasing factor (CRF) through its receptors (CRF_1 and CRF_2) is key component of the stress response. However, the mechanism by which the CRF system modulates the mPFC-BLA synapses in the stress response is not fully understood. CRF_1 modulate the stress response regulating the activity of mPFC neurons. However, the role of CRF_2 in PFC is poorly understood. Our results show that CRF_2 is expressed in BLA nerve terminals of the PFC. Electrophysiological and neurochemical approaches indicate that CRF_2 exerts an inhibitory role in the BLA-mPFC synaptic transmission.

INTRODUCTION

The medial prefrontal cortex (mPFC) is the main brain area that process information about cognition, motivation and emotion (1). The basolateral nucleus of the amygdala (BLA) is a key component of the network that regulates mPFC to process emotional information, such as stressful stimuli or fear (2, 3). The BLA information is transferred to layers 2 and 5/6 of the mPFC by excitatory afferents (4-6). The mayor connection of the BLA is to mPFC pyramidal neurons and only a slighter portion is to mPFC GABA interneurons (5, 7, 8). The BLA input to mPFC is glutamatergic, however it exerts a GABA-mediated fast feedforward inhibition of pyramidal mPFC (8, 9). The cellular and neurochemical basis of the BLA-mPFC circuitry linking emotion to decision making have not been fully identified.

Stress constitutes an important factor of the animal behavior that modifies mPFC function (10, 11). Interestingly, it has been demonstrated that acute stress exposure precludes BLA-PFC synaptic plasticity by an unknown mechanism (12). The corticotrophin releasing factor (CRF) system integrates both the endocrine and behavioral responses to stress, thus playing a pivotal role (13). The CRF system is has two G protein-coupled receptors, CRF_1 and CRF_2, which control the cellular excitability and synaptic plasticity in the brain (14-16) including mPFC (17-19). While the role of CRF_1 in mPFC is well-known, the function of CRF_2 still is uncovered (20). It has been shown that CRF and dopamine have synergistic effects upon BLA-mPFC EPSPs and that this interaction is modified by repeated exposure to cocaine (16). Collectively, these evidences prompted us to hypothesize that presynaptic CRF_2 fine tune BLA-mPFC transmission plasticity. To address this question, we determined the expression of CRF_2 in synaptic terminals of the BLA to mPFC and measured the impact of CRF_2 in the plasticity and glutamate release of the BLA-mPFC transmission. Interestingly, we demonstrated the presence of CRF_2 protein and mRNA in BLA nerve terminals and that the

antagonism of CRF_2 in mPFC increases LTP and glutamate release induced by the activation of the BLA to mPFC connection. Our results also show that the effect of the CRF2 antagonist is abolished in the presence of a D1R antagonist. Overall, our results show that CRF2 in mPFC nerve terminals originated in BLA plays a key role regulating the connection between these two key brain nuclei involved in relating emotional stress and decision-making control. Furthermore, our results reveal a significant interaction between CRF2 and D1R in the regulation of the BLA/mPFC synapse.

RESULTS

CRF_2 and D1R colocalize in mPFC glutamatergic nerve terminals from BLA

There is evidence of functional action of CRF_2 in mPFC (16), thus we aimed to determine the precise subsynaptic distribution of CRF_2 in a synaptosomal preparation devoid of postsynaptic elements (21). To this end, we performed CRF_2 immunofluorescence detection in mPFC synaptosomes enriched in presynaptic elements (Fig. 1), as demonstrated by co-staining with synaptic markers (Fig. 1A, 1B). Interestingly, CRF_2 was found in presynaptic mPFC nerve terminals colocalizing with D1R (Fig. 1A). Importantly, mPFC nerve terminals bearing CRF_2 and D1R were also positive for the vesicular glutamate transporter 1 (Vglut1) (Fig.1B). Thus, these results demonstrated that CRF_2 and D1R coexist in glutamate mPFC nerve terminals.

Based in the information reporting that certain proteins are synthetized directly in nerve terminals (22, 23), we aimed to demonstrate whether this was the case for CRF_1, CRF_2 and D1R. To this end, we extracted mRNA form mPFC synaptosomes and dissected tissue and analyzed the presence of specific transcripts by RT-PCR (Fig. 1C). Interestingly, while CRF_2 mRNA was found only in the mPFC synaptosomal preparation, D1R and CRF_1 transcripts were found in both in synaptosomes and whole tissue. To further control the quality of the mRNA samples, we analyzed the presence of the mRNA for RE1-Silencing Transcription factor (REST) (24, 25). REST mRNA was present only in dissected mPFC tissue but not in synaptosomes. Thus, our data supports the expression of both CRF_1 and CRF_2 in mPFC nerve terminals. Furthermore, our data show that CRF_2 are expressed in mPFC glutamatergic nerve terminals.

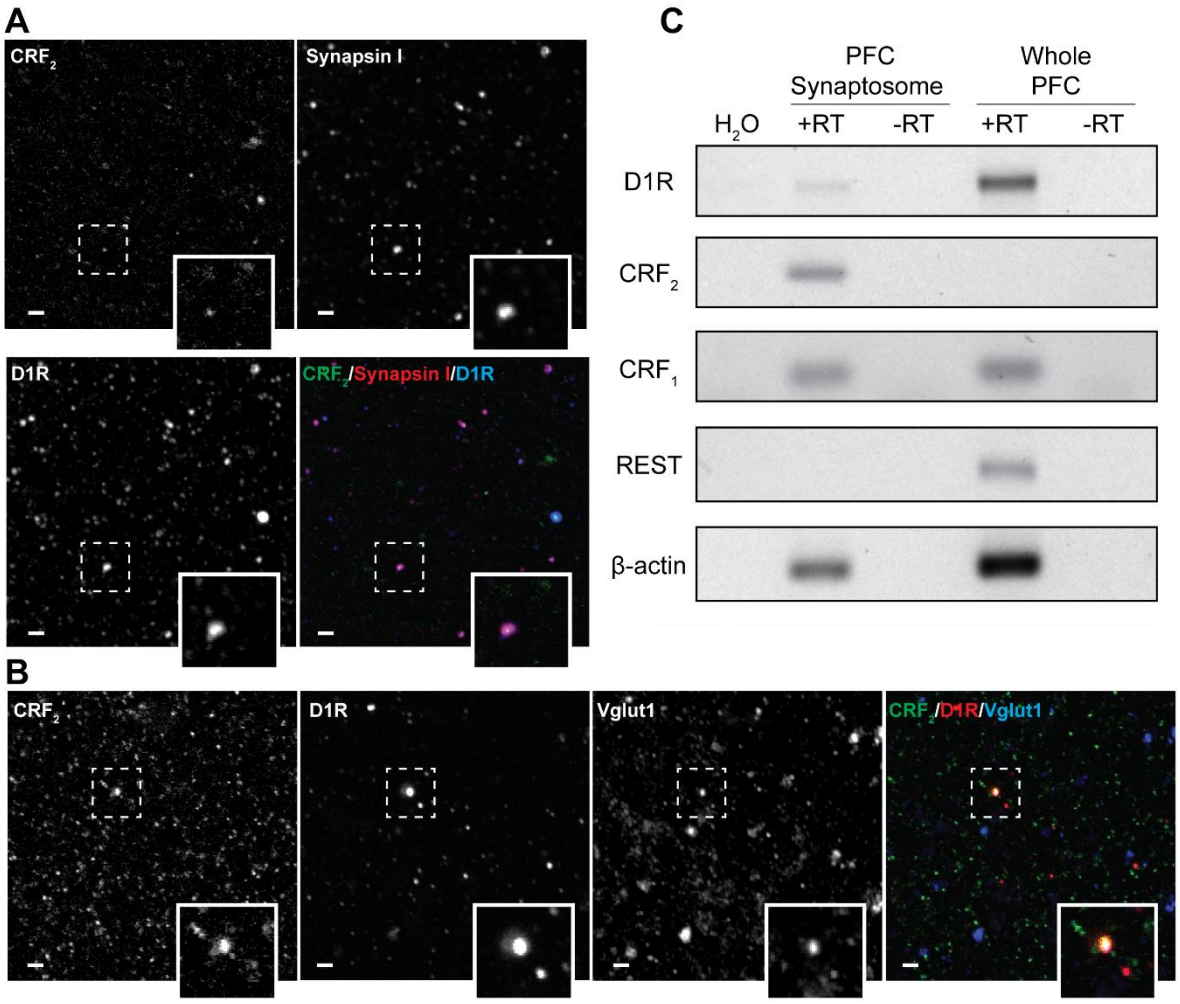

Figure 1. CRF_2 is expressed in presynaptic terminals of mPFC. (A and B) Confocal immunodetection of CRF_2 in a preparation of mPFC synaptosomes, devoid of presynaptic elements. (A) Immunofluorescence processed for CRF_2 (green), Synapsin I (red) and D1R (blue) (scale bar: 2 µm). (B) Immunofluorescence processed for CRF_2 (green), D1R (red) and Vglut1 (blue) (scale bar: 2 µm). The inserts of each image are a magnification of the square in the 100X image. (C) Reverse transcriptase polymerase chain reaction (RT-PCR) analysis for D1R, CRF_2, CRF_1, REST and β-actin in whole mPFC and in the mPFC synaptosomal preparation.

Next, we injected the anterograde tracer biotinylated dextran amine (BDA) in BLA to determine whether mPFC nerve terminals bearing CRF_2 are originated in the BLA. Fig. 2A shows the placement of the BDA injection in BLA and fig. 2B the labelling of nerve fibers in mPFC. Immunofluorescence in mPFC synaptosomes revealed the colocalization of CRF_2 with BDA (Fig. 2C). In addition, CRF_2 colocalized with BDA and D1R in some mPFC synaptosomes (Fig. 2C). Overall, these results suggest that CRF_2 are co-expressed with D1R in mPFC glutamatergic terminals originated in BLA.

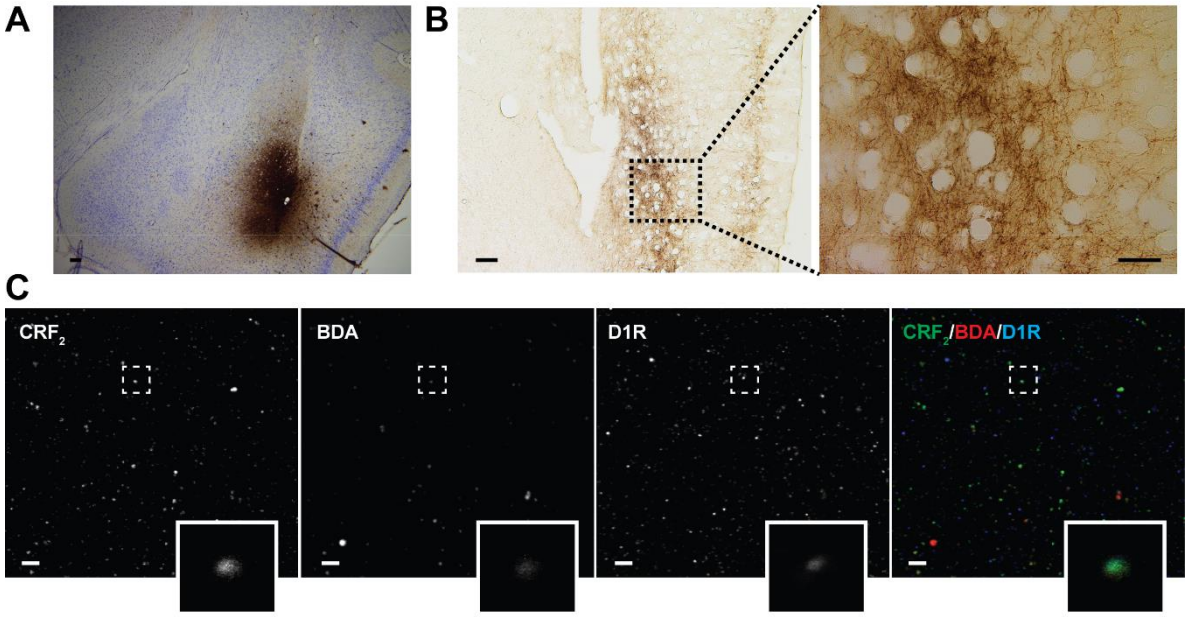

Figure 2. CRF_2 is expressed in mPFC synaptic nerve terminals originated in BLA. (A and B) Sample coronal brain sections showing the injection site of BDA in BLA (A) and the labelled BDA fibers in PFC (B) (scale bar: 100 µM, 100 µm and 200 µm respectively). (C) Confocal images of mPFC synaptosomes obtained from a BDA injected animal and subjected to immunofluorescence for CRF_2 (green), BDA (red) and D1R (blue) (scale bar: 2 µm). The inserts of each image are a magnification of the square in the 100X image.

CRF_2 negatively controls glutamate release in the BLA-mPFC circuit

Next, we aimed to determine the neurochemical consequences of CRF_2 expression in mPFC glutamatergic synaptic terminals. To this end, by means of microdialysis experiments we assessed mPFC glutamate release upon BLA stimulation in the presence or absence of local infusion of antisauvagine-30 (aSvg-30, 1 μM), through the microdialysis probe (Fig. 3A and D), a specific CRF_2 antagonist (26, 27). Interestingly, infusion of aSvg-30 in mPFC through the microdialysis probe significantly increased BLA-induced mPFC glutamate release (Fig.3A). In addition, the increase in mPFC extracellular glutamate level was also observed when kynurenic acid (Kyn, 1 mM) was mPFC infused through the microdialysis probe (Fig. 3A). These results suggested that CRF_2 negatively regulated BLA-induced mPFC glutamate release independently of mPFC glutamatergic activity. Importantly, this observation implies that CRF could be eventually present at mPFC controlling glutamate release through CRF_2. The quantification of CRF levels in mPFC total extracts and synaptosomes revealed its presence at low nanomolar concentrations (0.64 ± 0.15 (N=2) and 0.34 ± 0.15 nM (N=3), respectively.

Recently, it has been shown that CRF_1 in mPFC has a role in stress mediated responses (28), thus we evaluated the effect of mPFC infusion of CP154,526 (1 μM), a selective CRF_1 antagonist. Interestingly, CP154,526 did not affect BLA-induced mPFC glutamate release (Fig. 3B). Next, as we observed colocalization of CRF_2 and D1R in mPFC nerve terminals, and it has been shown that D1R activation regulates mPFC excitatory synaptic transmission in a complex manner (29, 30), we analyzed the effect of mPFC infusion of SCH23390 (1 μM), a D1R selective antagonist, BLA-induced mPFC glutamate release. Thus, infusion of SCH23390 alone did not affect basal and BLA induced mPFC glutamate release (Fig 3C). Interestingly, the co-infusion of SCH23390 with aSvg-30 blocked the activation observed

with aSvg-30 alone (Fig. 3C) revealing that CRF_2 and D1R significantly interact in the BLA/mPFC synapse.

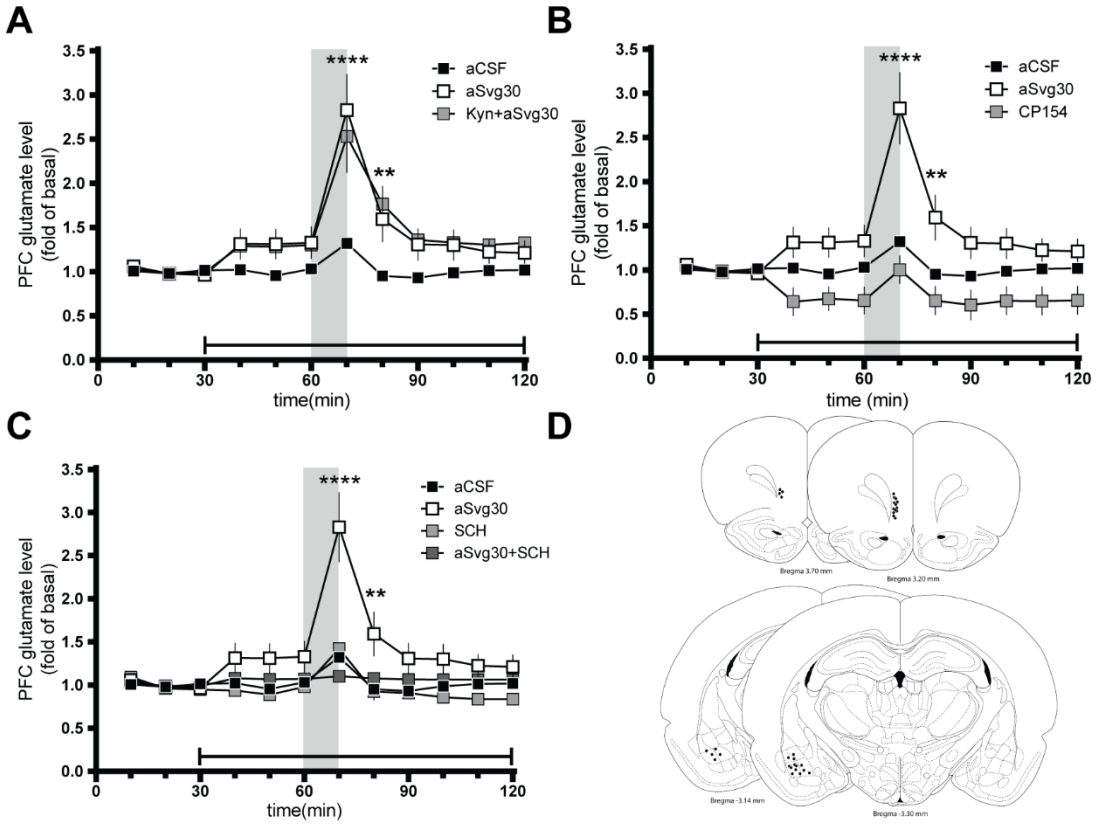

Figure 3. The presence of aSvg-30, specific CRF_2 antagonist, in the mPFC increases mPFC glutamate extracellular induced by the stimulation of BLA. (A, B and C) *In vivo* measurement of mPFC extracellular glutamate levels using *in vivo* microdialysis. Vertical gray bars indicate the time of BLA perfusion with 70 mM K^+-aCSF and horizontal black bars indicate the time of intra-mPFC infusion of each antagonist. (A) Intra-mPFC extracellular glutamate levels in the presence of 1µM aSvg-30 and 1mM Kyn (n = 3). (B) Intra-mPFC extracellular glutamate levels in the presence of 1 µM aSvg-30 and 1 µM CP154,526, specific CRF_1 antagonist (CP154, n = 3). (C) Intra-mPFC extracellular glutamate levels in the presence of 1 µM aSvg-30 and/or 1 µM SCH23390 (SCH; n = 3). (D) Brain coronal sections showing the placement of the microdialysis probes in BLA (-3.1 mm to -3.3 mm from bregma) and mPFC (3.7 mm to 3.2 mm from bregma). Two-way ANOVA followed by Bonferroni's multiple comparison post hoc test compared to the aCSF (****<p<0.0001; **

P<0.005). Values are expressed as mean ± SEM of the fold of the first 3 basal values of each condition.

CRF_2 inhibits *in vivo* mPFC LTP induced by electrical BLA stimulation

To further explore the functional role of CRF_2 in mPFC we assessed its contribution to the synaptic plasticity of BLA-mPFC transmission *in vivo* (31, 32) (Fig.4). Interestingly, we found that mPFC infusion of aSvg-30 (300 nM) robustly increased BLA-induced mPFC LTP in the early phases of the LTP (t5-10 and t15-20) (Fig. 4A, 4B). Consistent with the evidence obtained the microdialysis experiments, CP154,526 failed to modify BLA-induced mPFC LTP (Fig.4C, 4D). Finally, intra mPFC co-infusion of aSvg-30 and SCH23390 (10 µM) blocked the BLA-induced mPFC LTP produced by aSvg-30 (Fig.4E, 4F). Overall, CRF_2 has an inhibitory role in the synaptic plasticity between BLA and mPFC that it is not observed in the presence of a D1R antagonist.

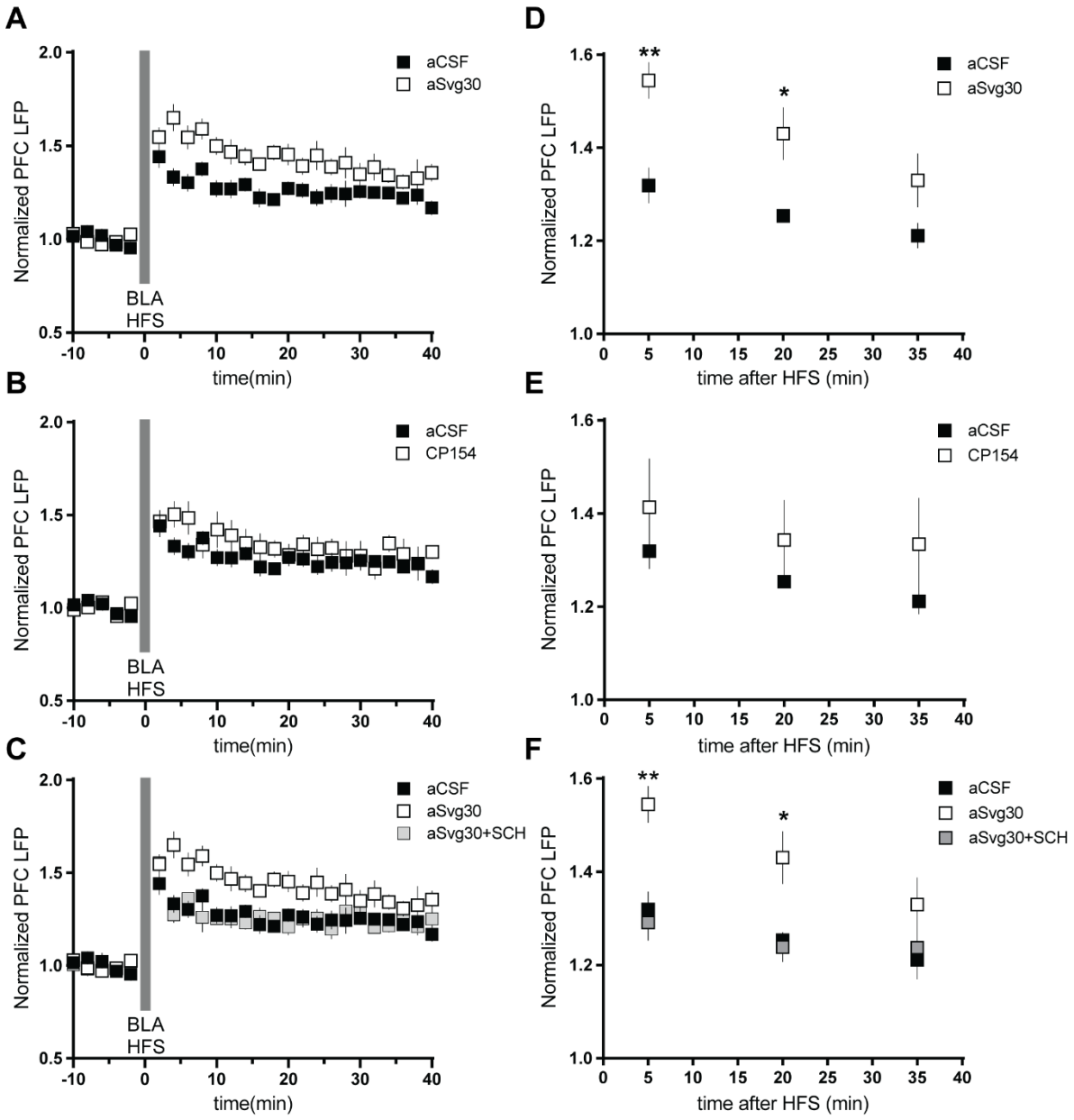

Figure 4. The presence of aSvg-30, specific CRF_2 antagonist, in mPFC increases *in vivo* local field potential (LFP) induced by the stimulation of BLA. (A-C) *In vivo* mPFC LFP recordings induced by electric stimulation of the BLA. Vertical gray lines indicate the time of application of high frequency stimulation (HFS) protocol in the BLA. (A) Relative to the aCSF controls (n = 5), local mPFC infusion of CRF_2 antagonist (aSvg-30, 300nM) (n = 6) induced a potentiation of the BLA-LTP. (B) Local mPFC infusion of CRF_1 antagonist

(CP154, 1 μM) (n = 6) not affected the BLA induced-LTP. (C) mPFC coinfusion of aSvg-30 (300 nM) and SCH23390 (SCH, 10 μM) (n = 6) blocked the potentiation in the BLA-induced LTP by aSvg-30 alone. (D-F) Summary mean LFP response obtained at early phase (5-10 min), middle phase (15-20 min) and late phase (35-40 min) of LTP post-HFS in the presence of the CFR_2, CRF_1 and D1R antagonists. Two-way ANOVA followed by Bonferroni's multiple comparison post hoc tests compared to aCSF (*$p<0.05$, **$p<0.005$). Values are expressed as mean ± SEM.

DISCUSSION

In the present study, we show for the first time the existence of CRF_2 in mPFC glutamatergic nerve terminals controlling the synaptic plasticity of the BLA-mPFC circuitry. We revealed that CRF_2 mRNA and protein are present in mPFC glutamatergic nerve terminals originated in BLA. Our data also show the co-existence of CRF_2 and D1R in mPFC nerve terminals originated in BLA and that an interaction between CRF_2 and D1R regulates glutamate release and the plasticity of the BLA/PFC glutamatergic connection.

CRF receptor express differentially in the brain (33). Presynaptic CRF_2 has been shown in glutamatergic nerve terminals of the lateral septum (14, 15) and in ventral tegmental area (VTA) GABAergic interneurons (34). Interestingly, while the expression and function of CRF_1 in mPFC has been described, (28, 35), the evidence for the existence of CRF2 in mPFC is still scarce. Our results clearly show that CRF_2 is expressed in mPFC glutamatergic nerve terminals originated in BLA. Interestingly, we found that CRF_2 protein as well as its mRNA is present in nerve terminals. It has been shown that the presence of tyrosine hydroxylase mRNA in axons and nerve endings plays a role in regulating the local level of the enzyme (36, 37). Thus, our results suggest that CRF_2 can be locally synthetized in nerve terminals, thus favoring an additional mechanism for regulating the amount of available CRF_2 in the PFC nerve terminals.

In this study, we show that CRF_2, but not CRF_1, has a local inhibitory effect on BLA-induced mPFC glutamate release. It has been described that CRF_1 increases spontaneous glutamate release in the central amygdala (38) and that activate pyramidal neurons in mPFC (28). The effects of CRF_2 over glutamate transmission are controversial. Thus, there is information reporting a CRF_2-mediated increase of VTA dopaminergic neurons excitability through a mechanism involving intracellular calcium release (39). In addition, it has been shown that

CRF, through CRF_2 activation, increases VTA glutamate release only in animals exposed to repeated doses of cocaine (40). Our evidence shows a clear pharmacological difference between both CRF receptors in mPFC. Hence, CRF_2, but not CRF_1, regulates BLA-induced mPFC glutamate release, thus supporting a role for CRF_2 controlling BLA-mPFC synaptic transmission. In addition, we also showed that the magnitude of the LTP in the synapses between BLA and mPFC is increased by a CRF_2 antagonist, but not by a CRF_1 antagonist. Thus, while CRF_1 does not modulate BLA-mPFC LTP, CRF_2 has an inhibitory role. Overall, these results concur with those in VTA showing that CRF_2 activation inhibits excitatory postsynaptic currents (EPSC), a mechanism involving presynaptic CRF_2 increasing GABA release and therefore decreasing VTA glutamate release (34).

Our data is consistent with an interaction between CRF_2 and D1R in the BLA-mPFC synapses. We found that both receptors are co-expressed in mPFC nerve terminals originated in BLA (Fig. 1B and 2C) and D1R blockade precluded CRF_2-dependent potentiation of BLA-induced mPFC glutamate release and LTP (Fig. 3C, 4E-F). Importantly, our data is consistent with previous studies showing that mPFC D1R blockade do not modify BLA-mPFC LTP (41). Thus, these results suggest that the dopamine-mediated action of D1R in mPFC depends in the present of CRF tone. Previously, it has been demonstrated a cocaine sensitive CRF_2-D1R interaction in mPFC (16). However, the inhibitory CRF_2 function in mPFC described here in has not been reported so far. Evidence suggesting that CRF_2 exerts a negative control on synaptic transmission working at the presynaptic level has been reported in other brain regions involving different mechanisms. It has been shown that CRF_2 mediates an increase in the GABAergic tone in VTA (34) and in the central nucleus of the amygdala (42) that results in a decrease in glutamate release. It has also been shown that CRF_2 inhibits P-currents in Purkinje neurons via Goα-dependent PKC epsilon pathway (43). Future studies will be

aimed at understanding the mechanisms by which the CRF/CRF_2 tone unmasks the action of D1R in the BLA to PFC neurotransmission.

BLA innervates mPFC pyramidal neurons with glutamatergic afferences (5, 6), however it drives feedforward inhibition of mPFC (9). It has been proposed that BLA projecting neurons activate mPFC GABAergic interneurons and that GABA would inhibit mPFC pyramidal neurons (5, 44, 45). Our results suggest that CRF through CRF_2 located in BLA glutamatergic afferences to mPFC could be key modulator of the BLA-mPFC neuronal transmission contributing to the state of inhibition of the mPFC by the BLA (8, 9).

In summary, we show that CRF_2 co-distributes with D1R in mPFC terminals originated in BLA and that inhibition of CRF_2 increases BLA-induced mPFC glutamate release. Furthermore, our data suggest that the tonic activation of mPFC CRF_2 could be masking the activity of dopamine upon D1R in mPFC or D1R that coexist with CRF_2 in mPFC nerve terminals coming from the BLA. Overall, our new findings should aid to better understand the role of the BLA-mPFC synapse in emotional disorders in which PFC GABAergic interneurons have some deficits, such anxiety disorders or schizophrenia and in disorders associated to changes in dopamine, such as addiction (46-50). Further studies should address whether the observed interaction between CRF_2 and D1R in the mPFC/BLA synapses is involved in the complexity of the role of mPFC in stress (51) and in the interaction between emotional stress and decision making.

Experimental Procedures

Animals

Male Sprague-Dawley rats (270–300 g) were used. The experimental protocols were approved by the Bioethical Committee of the Faculty of Biological Sciences of Pontificia Universidad Católica de Chile. Electrophysiological experiments were performed following the USPHS Guide for Care and Use of Laboratory Animals and were approved by the Rosalind Franklin University Institutional Animal Care and Use Committee for the care and use of laboratory animals.

Preparation of mPFC synaptosomes

Purified synaptosomes of mPFC, devoid of postsynaptic density, were prepared on a discontinuous Percoll gradient as described (21, 52, 53). The mPFC was dissected out of coronal slices of 4 animals following the Atlas of Paxinos and Watson (54). The extracted tissue was placed in a glass Potter homogenizer with 10 mM HEPES, 320 mM sucrose and 3 mM EDTA, pH 7.4, and centrifuged at 1000 g for 10 min at 4 °C. The supernatant was centrifuged at 17000 g for 20 min at 4 °C. The obtained pellet was re-suspended and centrifuged in a Percoll gradient (PVP-silica colloid; Sigma Aldrich, St Louis, MO, USA) at 15000 g for 20 min at 4 °C. The synaptosomal fraction was dissolved (in an equal volume to the fraction obtained) in 320 mM sucrose solution for immunofluorescence or RIPA lysis buffer (Millipore) solution for western blotting. The synaptosomal protein concentration was determined by Micro BCA™ Protein Assay Kit (Thermo Fisher).

Immunofluorescence in mPFC synaptosomes

Immunofluorescence in synaptosomes was performed as previously described (52, 53). Synaptosomes from mPFC (15μg of synaptosomal protein) were seeded on coverslips coated

with poly-L-lysine (Sigma Aldrich) and fixed with 4% PFA/ 10 % sucrose for 15 min, permeabilized with 0,2% Triton X-100 and incubated for 2 hours with blocking solution (4% bovine serum albumin (BSA) in PBS). The synaptosomes were incubated overnight at 4ºC with primary antibodies and thereafter for 2 hours with the secondary antibodies (1:200; Invitrogen). The primary antibodies used were: rabbit anti-synapsin I (1:500; SC-20780; Santa Cruz Biotechnology); goat anti-CRF_2 (1:200; SC-1826; Santa Cruz Biotechnology); rabbit anti-CRF_2 (1:200; NBP2-13875; Novus Biologicals); mouse anti-vesicular glutamate transporter 1 (1:500; 75-066; UC Davis/NIH NeuroMab Facility); rabbit anti-D1R (1:500; SC-14001; Santa Cruz Biotechnology); mouse anti-D1R (1:200; SG2-D1a; Novus Biologicals). The images were captured with a 100X objective in a confocal microscope (Olympus, Fluoview 1000) and with FLUOVIEW v6.0 software.

Biotinylated dextran amine injections and immunohistochemistry

Rats were anesthetized with isofluorane (4% for induction and 1-1,5% for maintenance) and stereotaxically injected with 1 µL of 10% Biotinylated dextran amine (BDA) 10 kDa (Thermo Fisher) at a rate of 0,1 µL/30s with a 33-G Hamilton syringe in the BLA (AP= 2.8 mm, ML=4.8 mm and DV=8.2 mm from Bregma). Seven days after the surgery, the animals were decapitated to prepare synaptosomes from the mPFC or perfused for immunohistochemistry. Animals were perfused intracardially with 4% PFA, brains were postfixed in 4% PFA overnight and then kept in 20 % sucrose for 48 h. Brains were sliced in 30 µm coronal sections with a cryostat (Leica CM 1510, Germany). BDA immunohistochemistry was performed as described (55). Coronal sections were mounted on gelatin-coated slides and coverslipped using Entellan® (Merck) and the images were captured in an epifluorescence microscope (Olympus). In the case of mPFC synaptosomes prepared

from BDA injected animals, they were processed for immunofluorescence using the secondary antibody Streptavidin-AlexaFluor^{647} (1:200; Thermo Fisher).

CRF quantification by ELISA

CRF in synaptosomal preparation and whole mPFC of controls rats was quantified using a commercial assay kit (CRF EIA kit S-1169; Peninsula Laboratories International, Inc., CA, United States). Data represent the averages of 2 independent experiments.

RT-PCR

Total RNA from whole mPFC and synaptosomal preparation from mPFC was isolated using Trizol reagent (Invitrogen). The reverse transcriptase enzyme used was the RevertAid Reverse Transcriptase (Thermo Fisher). The primers used are listed in Table 1. PCR was carried out with Platinum®Taq DNA Polymerase (Invitrogen) and the products were sequenced using Macrogene´s sequencing service (Macrogene, Seoul, Korea).

***In vivo* microdialysis**

Animals were anesthetized with 8% chloral hydrate (400mg/kg, i.p.) and placed in a stereotaxic apparatus. The body temperature was maintained by an electrical blanket at 37°C and the anesthesia was maintained at 0,8 µL/min by an electrical infusion pump (BASi). Microdialysis probes, 2 mm length (MAB 2.14.2, Microbiotech) were implanted in the mPFC (AP= 3.2 mm, ML=0.7 mm and DV=5.0 mm from Bregma), and CMA 11 (CMA Microdialysis AB) were implanted in the BLA (AP= 2.8 mm, ML=4.8 mm and DV=8.2 mm from Bregma). The microdialysis protocol was as previously described (55). Artificial cerebrospinal fluid (aCSF) was perfused through the microdialysis probes at 2 µL/min. After the stabilization period (90 min), every 10 min samples were collected from the mPFC. At the time indicated, 70 mM K^+-aCSF was perfused through the microdialysis probe in the

BLA during 10 min. Antisauvagine-30, (1 µM, Tocris), CP154526 (1 µM, Tocris), SCH23390 (1 µM, Tocris) and Kynurenic Acid (1 mM, Sigma) were perfused intra-BLA, through the microdialysis probe, when indicated in the figures. After the experiments, the brains were removed and store in 4%PFA for verification of the microdialysis probe placements. The determination of glutamate was performed using HPLC-fluorometric determination, as previously described (56).

In vivo recording of local field potentials (LFP) in the medial PFC induced by electrical stimulation of BLA

Animals were anesthetized with 8% choral hydrate (400 mg/kg i.p.) and fixed in a stereotaxic apparatus, as previously described (32). mPFC LFP responses elicited by the stimulation of the BLA were recorded with a concentric bipolar electrode (SNE-100x 50 mm; Rhodes Medical Instruments Inc.), amplified (Cygnus Technology Inc.), filtered (bandwidth, 1–100 Hz), and digitized (Digidata 1440A; Molecular Devices) at a sampling rate of 10 kHz. BLA stimulation was conducted with a concentric bipolar electrode (NE-100x, 50 mm), with every 15 s electrical pulses of 0.75 mA and 300 µs in duration through a computer-controlled pulse generator (Master-8 Stimulator; A.M.P.I.). A period of 10 min for stabilization and a second period of 20 min for stable baseline recording of LFP in mPFC were registered before delivering the high frequency stimulation protocol (HFS; 50 pulses at 100 Hz/15 s X 4) in the BLA. Afferent-driven plasticity in the mPFC was determined by the changes in the slope of the evoked mPFC-LFP in 40 min period post-HFS (32).

Antisauvagine-30 (1 µL at 300 nM, Tocris), and SCH23390 (1 µL at 10 µM, Tocris) were infused intra mPFC through a 28-gauge infusion cannula (length, 11 mm; Plastics One Inc.)

attached to the recording electrode. The rate of the infusion was 0.1 µL/min and the drugs were delivered 20 min before the baseline for the HFS protocol.

Statistical analyses

The statistical analyses were performed with the statistical software GraphPad Prism 6 (GraphPad Software). The data is expressed as the mean ± SEM. All plots in the LFP experiments are the normalization of the HFS drive LFP in the mPFC every 2 min. The microdialysis and HFS-driving LFP experiments were analyzed with two-way ANOVA followed by Bonferroni's multiple comparisons test.

Acknowledgements

The authors thanks Dr. Alon Chen for the thoughtful insight he gave us to further pursue our work. This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) (Grant 1150244). H.E.Y. was recipient of a doctoral fellowship from Comisión Nacional de Investigación Científica y Tecnológica (CONICYT), Chile.

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Table 1. Primers used for RT-PCR

GENE	Forward primer	Reverse primer
D1R	5´-CATGCCAAGAATTGCCAGACC 3´	5´-CTCTTCCTTCTTCAGGTCCTC-3´
CRF_2	5´-AAGAGCTGCTTTTGGACGGCT-3´	5´-GGCGATTCGGTAATGCAGGTC-3´
CRF_1	5´-TCCACCTCCCTTCAGGATCA-3´	5´-TGCAGGCCAGAAACATTGC-3´
REST	5´-TACACGGCACACCTGAAGCACC-3´	5´-TTGCGTGTCGGGTCACTTCGTG-3´
β-Actin	5´-CACCCGCGAGTA CAACCTTC-3´	5´-CCCATACCCACCATCACACC-3´

DISCUSSION

In the present thesis, we provide additional information of the CRF system. This is the first study showing that CRF2 modulates the BLA transmission to the mPFC, and we elucidate how CRF2 and D1R agonists modulate the interaction between CRF2 and D1R. We showed that CRF2 and D1R are expressed in PFC glutamatergic pre-synaptic nerve terminals that are originated in the BLA. Even more, our results showed that the CRF mRNA is localized in nerve terminals of the mPFC. In addition, we showed that CRF2 negatively modulates synaptic transmission and glutamate release of BLA-to-mPFC, and that inhibiting D1R in the synaptic transmission of BLA-mPFC blunts this modulation. Additionally, we showed that the interaction between CRF2 and D1R decreased the presence of CRF2 in the plasma membrane, in the presence of dopamine. The treatment with dopamine and CRF significantly increased the interaction between CRF2 and D1R. Finally, we showed that dopamine and CRF increased ERK signaling when CRF2 and D1R are co-expressed. Interestingly, the co-treatment with CRF and dopamine did not increase the phosphorylation of ERK protein. These results give a key role to CRF2 modulating the synaptic connection between BLA and mPFC; and give a possible role to the heteromer CRF2/D1R modulating the BLA-mPFC synapses.

Through RT-PFC (Fig. 1C, chapter II) we detected CRF2 mRNA in mPFC synaptosomes devoid of post-synaptic density and through immunofluorescence in mPFC synpatosomes from animals injected with BDA in the BLA, we detected the protein for CRF2 and D1R in PFC synaptosomes originated in the BLA (Fig. 2C, chapter II). These results are consistent with previous data showing functionality of CRF2 in the mPFC (Orozco-Cabal et al., 2008;

Guan et al., 2014) and complement the information about the expression of CRF receptors in the mPFC (De Souza et al., 1985; Lovenberg et al., 1995; Van Pett et al., 2000).

CRF receptors are differentially expressed in the brain (Van Pett et al., 2000). Presynaptic CRF2 has been shown in glutamatergic nerve terminals of the LS (Liu et al., 2004; Liu et al., 2005) and in VTA GABAergic interneurons (Williams et al., 2014). Interestingly, while the expression and function of CRF1 in mPFC has been described, (Uribe-Mariño et al., 2016; Van Pett et al., 2000), the evidence for the existence of CRF2 in mPFC is still scarce. Our results clearly show that CRF2 is expressed in mPFC glutamatergic nerve terminals originated in BLA (Fig. 1 and 2, chapter II). Interestingly, we found that CRF2 protein as well as its mRNA is present in PFC nerve terminals (Fig. 1, chapter II). It has been shown that the presence of tyrosine hydroxylase mRNA in axons and nerve endings plays a role in regulating the local level of the enzyme (Gervasi et al., 2016; Jimenez et al., 2002). Thus, our results suggest that CRF2 can be locally synthetized in nerve terminals, thus favoring an additional mechanism for regulating the amount of available CRF2 in the PFC nerve terminals. Thus, the possibility that CRF2 are present in other types of nerve terminals (Pickel, 2000; Carr and Sesack, 2000) could not be discarded and should be further explored.

Using electrophysiologic and *in vivo* microdyalisis approaches we found that D1R blocks the CRF2 potentiation of BLA- induced mPFC glutamate release and LTP (Fig. 3C; Fig. 4C, F, chapter II). Our results are consistent with an interaction of CRF2 and D1R in the BLA-to-mPFC synaptic transmission. Interestingly, our data (Fig. 3C, chapter II) is consistent with previous information showing that D1R blockade did not modify BLA-mPFC synaptic transmission (Flores-Barrera et al., 2014). Our results suggest that the D1R effects on the BLA-mPFC synapses depend in the presence of a CRF tone on the mPFC, because the D1R

effects is only perceptible when CRF2 was antagonized (Fig. 3C; Fig. 4C, F, chapter II). A CRF tone in the mPFC has been described in stressed situations (Meng et al., 2011) and has been described an inhibitory role of stress over the BLA-to-mPFC synaptic transmission (Maroun and Richter-Levin, 2003). However, the inhibitory CRF2 function in mPFC described here in has not been reported so far. Evidence suggesting that CRF2 exerts a negative control on synaptic transmission working at the presynaptic level has been reported in other brain regions involving different mechanisms. It has been shown that CRF2 mediates an increase in the GABAergic tone in VTA (Williams et al., 2014) and in the central nucleus of the amygdala (Fu and Neugebauer, 2008) that results in a decrease of glutamate release. It has also been shown that CRF2 inhibits P-currents in Purkinje neurons via Goα-dependent PKC epsilon pathway (Tao et al., 2009).

The interaction between CRF2 and D1R described herein and in the work of Orozco-Cabal et al (2008), could be explained by the reported interaction of CRF2 and D1R proteins (Fuenzalida et al., 2014). To learn about the pharmacological consequences of the interaction between CRF2 and D1R, we used a heterologous expression system. We used the α splice variant of CRF2 because is the most abundant in the brain (Dautzenberg and Hauger, 2002; Hauger et al., 2006). In figure 2 of the chapter I, the incubation of HEK293t cells in the presence of dopamine reduced the amount of CRF2α in the cell surface of HEK293t cells co-expressing CRF2α and D1R. This means that the stimulation of D1R is not capable of increasing the amount of the CRF2α partner in the cell surface. Furthermore, our data showed that the stimulation of D1R reduces the amount of CRF2α in the cell surface (Fig. 2, chapter I). It appears that D1R internalized CRF2α by the action of dopamine and that the internalized CRF2α remains intracellularly after the incubation with dopamine. Some GPCRs, after

incubation with their agonist, form a stable complex with other proteins (like β-arrestin) that impedes receptor´s resensitization (Oakley et al., 2001; Thomsen et al., 2016). The observation that D1R was internalized with CRF2α by the action of dopamine incubation (Fig. 2, chapter I), tells us that the CRF2α/D1R heteromer is stable enough to internalize both receptors using only the agonist for one of the heteromer components. It has been shown that the interaction between D1R with CRF2α is stable enough to change the cellular localization site of CRF2α by using a nuclear localization signal on D1R (Fuenzalida et al., 2014).

Growing evidence shows that GPCRs are capable of modulate the interaction of distinct proteins with their ligands (Magalhaes et al., 2012). In the current study, we observed that CRF2α and D1R did not modify their interaction by the dopamine treatment (Fig. 3, chapter I). Furthermore, our results show that the co-treatment with CRF and dopamine increased the amount of CRF2α in the immunoprecipitated, which means that the interaction between CRF2α and D1R heteromer increased in comparison with the control situation (Fig. 3, chapter I). This suggest that the internalization of CRF2 and D1R in conjunction increased the association with others heteromer partners. This phenomenon has been previously described for CRF1 (Magalhaes et al., 2010). Treatment with a CRF1 agonist increased the interaction of CRF1 with 5-HT2 receptor increasing the localization of the receptors in the plasma membrane. Our results showing that agonist treatment increased the amount of CRF2α/D1R heteromer formation (Fig. 3, chapter I) and decreased the amount of heteromer in the plasma membrane (Fig. 2, chapter I) suggest that the localization of the CRF2 and D1R determines the levels of heteromeric interaction.

The GPCRs could modulate the pharmacological properties of their signal transduction (Terrillon and Bouvier, 2004). We showed that dopamine can activate ERK 1/2

phosphorylation in cells transiently expressing D1R (Fig. 4C, D, chapter I). According with some data, the increasing activation of ERK 1/2 occurred in early phases of the agonist incubation (Navarro et al., 2010; Fiorentini et al., 2011). Dopamine and CRF increased significantly ERK 1/2 activation in cells expressing D1R and CRF2α (Fig. 5, chapter I). Both, CRF-activation and dopamine-increased ERK 1/2 phosphorylation were counteracted by CRF2α or D1R antagonists (Fig. 5, chapter I). The modulation of an antagonist of one of the receptors in a heteromer to block signals originated by the stimulation of the other receptor is a characteristic described for other heteromers (Ferrada et al., 2009; Navarro et al., 2010; Navarro et al., 2015). Furthermore, we also found mutual agonistic interaction between D1R and CRF2α on ERK 1/2 activation in transfected cells with D1R and CRF2α. The CRF2α agonist increased ERK 1/2 phosphorylation and this increase was counteracted when the agonist of D1R was co-incubated in HEK293t cells expressing D1R and CRF2α (Fig. 5, chapter I). It has been described a "cross-talk" for the signaling pathways of some GPCR heteromers, such as we described here for the CRF2α/D1R heteromer (Navarro et al., 2010; Navarro et al., 2015).

The modulation of the BLA-to-mPFC synapses by CRF2 and D1R suggests that the heteromer between CRF2 and D1R could modulate the neurotransmission between the BLA and mPFC synapses. It has been described that the heteromeric interaction between CRF1 and orexin receptor 1 (OX1) modulates VTA dopamine release (Navarro et al., 2015). This provides a new perspective of how the heteromeric interaction between GPCRs modulates the neurotransmitter homeostasis.

In summary, our results provide new relevant neuroanatomical and functional knowledge about the expression and functionality of CRF2 and D1R in the mPFC (Fig. 2). In addition,

our results delineate how the agonist for CRF2 and D1R modulate the heteromeric interaction and their signaling. Furthermore, in this work we provide evidence for the anatomical evidence summarized in figure 2, in which we show the location and effect of CRF2 in the mPFC and its possibility of interaction with D1R in the BLA-mPFC synapses. Further studies considering these new data are needed to determine the mechanism by which CRF2 could inhibit the synaptic transmission of BLA-to-mPFC and how the effect of the CRF2 in the mPFC could modulate some behaviors related to the BLA-mPFC transmission, like fear or emotion consolidation.

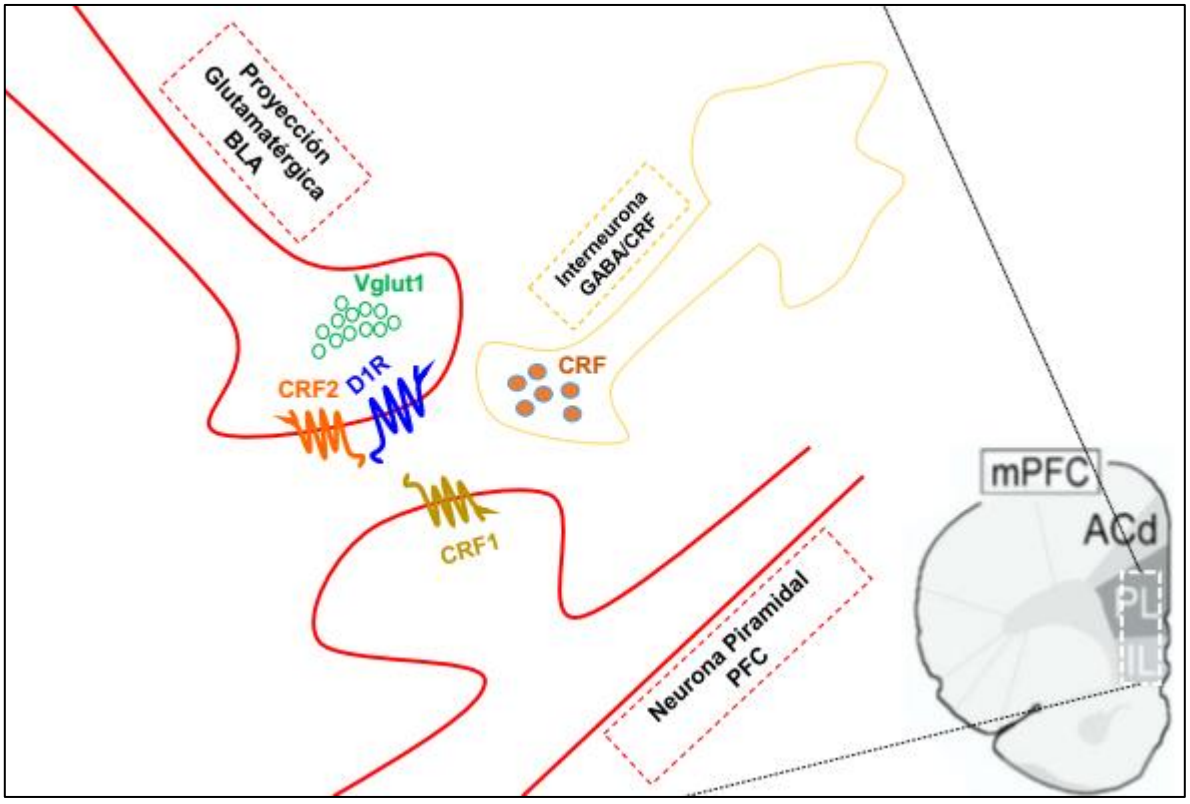

Figure 2. Summary of the anatomical evidence described in the thesis for CRF2 and D1R in the mPFC. The CRF peptide are expressed in the mPFC GABAergic interneurons and the CRF1 are expressed in the mPFC pyramidal neurons. Some of the CRF2 and D1R are expressed in the BLA glutamatergic terminals in the mPFC.

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