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MÁSTER EN INVESTIGACIÓN EN CEREBRO Y CONDUCTA

" EFFECTS OF BRAIN INSULIN IN CONDITIONED PLACE PREFERENCE USING EXERCISE AS A REWARD STIMULUS"

TRABAJO FINAL DE MÁSTER

Autora: Verónica Espinosa Fernández DNI: *20478003E*

Directora: Ana María Sánchez Pérez DNI: 19102961N

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ABSTRACT

Researches suggest that physical activity is effective to prevent or treat stress-related psychiatric disorders such as depression, anxiety, and substance abuse disorders. Both, natural and drugs rewards is processed by the mesolimbic dopamine system; and also this is implicated to reward-related behavioral disorders. Studies supports the hypothesis that physical activity act as powerful natural stimulus of the mesolimbic system, using the conditioned place preference (CPP) paradigm with cues associates to voluntary exercise on the running wheel. On the other hand, several studies had reported that insulin can modulated aspects related to memory and learning, which are involved in reward-related behaviors, however, further data are necessary to understand these mechanisms. In addition, an important mediator of insulin/IGF1 (insulin-like growth factor-1) signaling is ERK (extracellular signal–regulated kinases). Evidence supports its implication in learning and memory, and is expressed in the NAcc and activated during exposure to natural and drugs rewards. Our hypothesis is that if insulin signaling can modulate preference to drugs via its effect on dopaminergic transmission in the mesolimbic system, it could similarly modulate preference to the context paired with voluntary exercise. In order to set up the behavior paradigm to study insulin/IGF1 signaling effect in preference for a rewarding stimulus such as exercise, male Wistar rats intraventricular insulin injections treated were used to study the rewarding effects on CPP paired to voluntary wheel running. pERK, IRS2, and IRS1 expression was assessed by immunohistochemical analysis. We found that intraventricular insulin injection showed no significant differences on conditioned place preference using exercise as a reward stimulus. pERK phosphorylation increases in specific brain regions of the insulin injected rats. IRS1 and IRS2 expression displays a different pattern in prefrontal cortex nucleus accumbens and ventral tegmental area. We suggest that the responses of animals in a CPP paradigm can be improved by insulin, due to insulin signaling into the reward areas. Data of immunohistochemical analysis support other studies that reported role of insulin into VTA neurons. Nevertheless, are needed additional research focus on the effects of insulin injections into mesolimbic regions as NAcc or VTA to understand what it is the role of insulin modulating reward-related behaviors.

Key words

Insulin; mesolimbic dopamine system; reward; physical activity; conditioned place preference

1. INTRODUCTION

A healthy lifestyle includes regular physical exercise, and clinical evidence supports that physical activity is effective to prevent and even treat habitual stress-related psychiatric disorders including depression, anxiety and substance abuse disorders (Greenwood et al. 2011). Drug addiction is usually associated to alterations within the brain reward system, the mesolimbic circuit (Oliveira et al. 2009; Nestler & Carlezon 2006; Feltenstein & See 2009). The mesolimbic circuit that underlies the natural rewards is the main target of abuse and non-abuse drugs. Interestingly, repeated exposure to drugs or natural rewards induce plasticity in the mesolimbic pathway (Greenwood et al. 2011)(Lee et al. 2005). The mesolimbic pathway involves dopaminergic neurons in the ventral tegmental area (VTA) that project to the nucleus accumbens (NAcc), which is part of the ventral striatum. However, other regions also may be involved.

Studies with animals models supports the hypothesis that physical activity acts as powerful natural stimulus of the mesolimbic circuit, using the conditioned place preference paradigm (see below) with cues associated to voluntary exercise on the running wheel (Belke & Wagner 2005; Lett et al. 2000; Lett et al. 2001; Foley & Fleshner 2008; Iversen 1998).

Growing evidence has shown that insulin has a wide range of molecular targets in the central nervous system (CNS). Studies suggest that insulin is implicated in a multitude of brain functions, such as learning, memory, cognitive function, appetite, and others. If this is so, it is logical to think that failures in insulin signaling can lead to different types of brain disorders. Until the last years it was believed that insulin only entered the CNS through a blood-brain barrier (BBB), but there are studies that suggest the possibility of endogenous production, however, has not yet been sufficiently investigated. So, further studies are clearly needed to better understand insulin transport into the brain in health and disease (Gray et al. 2014; Heni et al. 2015).

Evidence at the last decades, have found a role of the insulin in food intake, plasma glucose levels and brain pathways associated with reward (Greenwood et al. 2011; Labouebe et al. 2013; Garcia et al. 2005; Galici et al. 2003; Figlewicz et al. 2006; 2008; Davis et al. 2010). Moreover, insulin signaling has an important role regulating

memory and cognition; which are processes also involved in the rewards-directed behaviors, among others. In fact, insulin signaling can affect areas involved in reward recognition (Gray et al. 2014; Craft et al. 2012; Davis et al. 2010; Pardini et al. 2006a; Samandari et al. 2013), although the mechanisms are not well understood. Regarding to reward-related behaviors, Labouèbe et al., 2013 reported data about the insulin in the VTA inhibited food anticipatory behavior and CPP for food, suggesting that insulin may attenuate the salience of food-related contexts or cues (Labouebe et al. 2013). In addition, Figlewicz and collaborates contribute in this subject, they suggested that effects of insulin on the mesolimbic dopamine system can interact with limbic system to diminish the reward value of stimuli ((Figlewicz 2003; 2006; 2008). Insulin can interact to the actions drugs into the CNS; there is converging evidence that this occurs through insulin regulation of the dopamine (DA) transporter (DAT), such as AMPH or methamphetamine (METH) psychostimulants drugs, which related DA neurotransmission. DAT activity decreases in rats with low blood insulin levels (Samandari et al. 2013; Owens et al. 2005; Galici et al. 2003; Sevak et al. 2009). Moreover, this modulation of insulin into the brain can developed a long-term plastic changes at the molecular level, such as a DAT function (Owens et al. 2005). Some authors demonstrated that insulin can presynaptically enhance the function of cocaine sensitive DAT into the nucleus accumbens, and may decrease impulsive behaviors (Schoffelmeer et al. 2011). Taken together, these data, offer some highlights to understand insulin-signaling effects into, mainly, the mesolimbic dopamine system and their adjacent regions that involved the reward-related behaviors.

1.1 Mesocorticolimbic or "Reward" System

Reward is a process that involves multiple neuropsychological components simultaneously: 1) hedonic pleasure, 2) the motivation that emerges by obtaining the reward and 3) associative learning. Therefore the neurobiological processes that underlie the reward system encompass emotion, motivation, learning and memory related to certain stimuli that have some relevance to the individual. The set of brain networks or structures of this system, also known as the mesocorticolimbic system of dopamine (DA), are responsible for the mechanisms that drive emotional and motivational regulation behavior.

Figure 1.A illustrates the VTA dopaminergic neurons outputs to the prefrontal cortex (PFC), central amygdala, basolateral amygdala (BLA), hippocampus, and others. These structures form a complex association through of their glutamatergic and GABAergic connections (Russo & Nestler 2013). In human brain, DA is synthesized by (VTA) neurons and substantia nigra (SN), these areas project to the ventral striatum (SV) includes nucleus accumbens, prefrontal cortex (CPF), the limbic system and the hypothalamus (Arias-Carrión et al. 2010)(Fig. 1B). These structures have been extensively studied; early experiments placed electrodes for brain self-stimulation in rat forebrain release of dopaminergic neurotransmitters was postulated to be due to the reinforcement by the press lever behavior. By mistake, the researchers had implanted the electrodes in the VTA, which fibers reach the nucleus accumbens (they thought they had inserted it in the septum). They observed that rat continued to self-stimulated, and preferred it to eat or drink despite adverse consequences. In, this experiment, the authors interpreted that they had located the center of pleasure (Olds & Milner 1954).

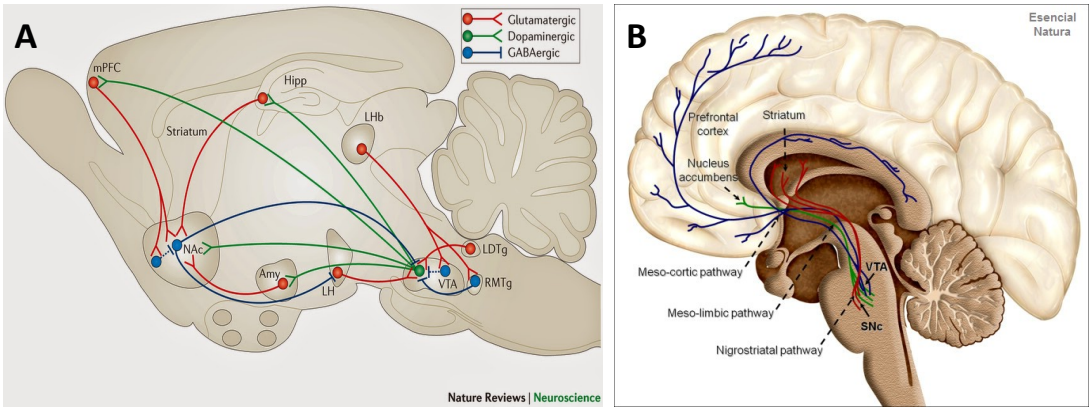

Fig. 1. Mesocorticolimbic circuit*. (A) Schematic glutamatergic, dopaminergic and GABAergic pathways and their connections on several regions of rat brain. (B) Mesocorticolimbic structures in the human brain. Dopaminergic neurons are located in the midbrain structures substantia nigra (SNc) and the ventral tegmental area (VTA). Their axons project to the striatum (caudate nucleus, putamen and ventral striatum including nucleus accumbens), the dorsal and ventral prefrontal cortex.*

To study the reward systems in an animal model the behavioral paradigm Conditioned Place Preference (CPP) is used. CPP analyzes the association of a given rewarding stimulus with a context. The paradigm starts with a habituation period

where the animal associates a context (conditioned place) to a reinforce stimulus (e.g. drugs of abuse, sweetened food) that alters the subject physiological state. The distinction of the contextual cues can be based on different sensorial modality (odoriferous, tactile or visual). The greater the dissimilarity between environments the easier the discrimination between them will be. The dependent variable is usually the residence time in both contexts. A preference for the conditioned place will be assumed when the time in the conditioned place is above the 60% of total test time. This phenomenon is usually attributed to the reinforcing properties of the stimulus. This paradigm follows the principles of classical pavlovian conditioning, where the reinforce stimulus is the unconditioned stimulus, (US) and the context, the conditioned stimulus (CS). Due to the association between these two stimuli (US and CS) the CS elicits the same physiological responses as the US. In other words, the context (CS) would acquire the ability to elicit approximation or avoidance behavior as the reinforce itself (US) (Prus et al. 2009).

1.2 Reward System Modulated by Insulin

Insulin is a hormone released by pancreatic beta cells in response to elevated levels of glucose in the blood. Moreover, insulin regulates energy expenditure and food intake. The insulin action is mediated by intracellular signaling cascades, which the receptor is activated by kinase Tyr and serine / threonine (Ser / Thr) action. Once the receptor is activated, a phosphate molecule is combined with tyrosine, which can lead to different intracellular cascade signaling: 1) pathway phosphatidylinositol 3-kinase (PI3K) associated with the glucose and lipid metabolism, and / or 2) pathways of mitogen-activated kinases (MAP kinases) involved in the protein synthesis (Reyes Olivares & Arellano Plancarte 2008).

Besides the well known metabolic effects, insulin and IGF-1 signaling has important role regulating memory and cognition (Sarah M. Gray, Rick I. Meijer 2014). Moreover, insulin/IGF-1signaling can affect areas involved in reward recognition (Craft et al. 2012; Davis et al. 2010; Pardini et al. 2006a; Samandari et al. 2013; Gray et al. 2014), however, the molecular mechanisms are largely unknown.

Previous studies showed that DA and insulin systems work together directing reward-related behaviors. Homeostasis of DA is modulated by the action of its

transporter (the dopamine transporter or DAT), which regulates the DA levels in the extracellular space. Insulin signaling can regulate DA neurotransmission (Daws et al. 2011a).via phosphatidylinositol-3-kinases (PI3K) or protein kinase B (PKB)(Akt) pathway, affecting DAT activity. Moreover, reduced insulin signaling decrease the responses to amphetamine (AMPH) consumption of (Galici et al. 2003; Bayat & Haghparast 2015; Samandari et al. 2013).

Insulin receptors (INS R) are expressed in NAcc and caudate putamen (CPu), brain regions that mediate the rewarding effects of both natural and drugs rewards (Kelley & Berridge 2002). INS R are also expressed by DA neurons of the VTA and pars compact substantia nigra (SNc) (Figlewicz et al. 2003; Stouffer et al. 2015).For example in caloric restriction or food deprivation conditions, insulin levels are decreased, and this reduction augments food seeking behaviors (Davis et al. 2010; Pardini et al. 2006a). Taken together, these data suggests that impaired insulin/IGF-1 signaling could link the co-morbidity between substance abuse and eating disorders. (Carvelli et al. 2002; Garcia et al. 2005; Davis et al. 2010; Daws et al. 2011b; Pardini et al. 2006a).

The mesolimbic dopamine system can suffer plastic changes by the action by repeated exposure to drugs (Kelley & Berridge 2002). Iñiguez et al., 2008 investigated the influence of IRS2 in the VTA on behavioral response to cocaine by selectively increasing or blocking IRS-2 expression. They found that overexpression of IRS-2 enhanced cocaine-induced behavioral responses, whereas blockade of IRS-2 deteriorated them (Iñiguez et al. 2008), suggesting that insulin signaling pathway in the VTA is required for cocaine action. Others studies, investigating the effect of insulin signaling in hedonic feeding, reports that insulin injection in the VTA decreases the motivational salience of contextual cues associated with food reward in rodents which were satiated, but does not alter hungry feeding. This results suggests that, separate from their expression in hypothalamic centers, the insulin modulates the rewarding aspects of feeding behavior, and regarding to this assumption it seems logical to think that it may serve as pharmacological therapy in the treatment of eating disorders in which the insulin signaling is impaired. Moreover, insulin signaling in mesolimbic areas has been shown to reduce the motivation for sucrose in cocaine-treated rats, and the preference for the context associated with palatable food (Labouebe et al. 2013; Mebel et al. 2012). In contrast, insulin seems to enhance CPP for cocaine ((Iñiguez et al. 2008). These data suggest that insulin plays an important

role in the control of inhibitory control behaviors due to its relationship with the dopaminergic system.

1.3 Physical Exercise as a Reinforcer of the Reward System

The reinforcing properties of stimuli such as food or sex have great relevance for survival. In contrast, the reinforcing qualities of secondary rewards suggest that humans, and most mammals, have the ability to assign conceptual value to wide range of events and behaviors that, in a way, may be considered as abstract. Unlike primary rewards, these secondary rewards, being physical activity an example, may have little relevance to survival,

Voluntary physical activity, as a natural reinforce, could be used to help in treatments of stress-related psychiatric disorders including depression, anxiety and substance abuse disorders. the mesolimbic reward system is therefore a potential target to prevent them (Greenwood et al. 2011). The "gratifying" property of the voluntary exercise has been extensively investigated with the CPP paradigm. Rodents show preference to the context paired with the post-effect of running on the wheel (Lett et al. 2000; 2001; Novak et al. 2012) indicating an association between the exercise (US) and the context (CS) (Belke & Wagner 2005; Brené et al. 2007).

In addition, natural stimuli such as sugar in a chronic intermittent treatment are rewarding and can induce plastic changes in the mesolimbic dopaminergic system (Spangler et al. 2004). The reward brain structures are responsible for the adaptation to reinforcing stimuli. Dopaminergic dysfunctions in the mesocorticolimbic pathways result in reward system impairments. These alterations underlie psychopathological problems such as drug addiction or mood disorders. In addition, accumulated evidence suggests that insulin and IGF-1 signaling are implicated in alterations related to reward system disorders including drug addictions, via DAT regulation. However, the mechanism by which insulin signaling affects these systems is largely unknown.

As previously discussed, the reward-related behaviors are complexly modulated and these involving the NAcc and the dopaminergic innervation from the VTA. The plastic changes at circuit level that underlie reward learning have received extensive attention, however, the molecular level that mediate such adaptations remain unclear. In

addition, an important mediator of Insulin/IGF1 signaling is ERK (extracellular signal–regulated kinases or classical MAP kinases). Mitogen-activated protein kinase (MAPK) amongst other functions, is implicated in learning and memory, then, that is expressed in the NAcc and activated during exposure to drugs such as cocaine and amphetamine (Valjent et al. 2004; 2005; Day 2008). ERK is activated by glutamate action in striatal neurons. Evidence suggests that ERK phosphorylation in the NAcc requires co-activation of NMDA glutamate receptors and D1 dopamine receptors. This gives ERK a role in detecting the functional relation between glutamatergic inputs and the possibility of obtaining a reward provided by dopaminergic neurons (Valjent et al. 2005; Schultz et al. 1997; Girault et al. 2007; Zamora-Martinez & Edwards 2014; Edwards et al. 2011). Furthermore, using CPP paradigm suggested that NAcc DA-mediated learning depends on MAPKs activity in AMPH-treated rats. Inhibitors of ERK and p38 MAPK impaired NAcc amphetamine-produced CPP. Consistent with previous research, they showing a role for MAPK in memory and plasticity in hippocampus and amygdala, and they fit the role of MAPKs in cellular cascades in the striatum or NAcc initiated by the activation of DA receptors (Gerdjikov et al. 2004). Psychoactive drugs, such as cocaine increased ERK phosphorylation in NAcc, PFC, and other regions, through a DA-dependent mechanism since DA-D1R (dopamine D1 receptors) antagonism blocks this effect (Valjent et al. 2004; Girault et al. 2007).Wheel running reduced drug seeking behavior, and this behavior was associated with higher pERK levels in the PFC in rats that showed abstinence symptoms to cocaine addiction than saline controls (sedentary and exercising rats, which have never had contact with cocaine) (Lu et al. 2006; Koya et al. 2009a; Lynch et al. 2010).

Taken together, if both glutamate and dopamine are upstream regulators of ERK to both natural (Shiflett et al. 2008) and drug (Lu et al. 2006; Zamora-Martinez & Edwards 2014; Edwards et al. 2011) rewards, pERK a suitable marker to study brain areas involved in the preference of animals to the context associated to the running wheel.

2 HYPOTHESIS and OBJECTIVES

The hypothesis of this study is that if insulin signaling can modulate preference to drugs via its effect on dopaminergic transmission in the mesolimbic system, it could similarly modulate preference to the context paired with voluntary exercise.

To prove the hypothesis the following objectives were proposed:

1. To establish the CPP paradigm with voluntary exercise as a reinforcer, or positive stimulus
2. To establish the protocol of icv insulin injections to evaluate the insulin effects on CPP behavior
3. To quantify ERK activity after CPP paradigm as a marker for insulin action in brain areas of the mesolimbic circuit
4. To study the expression of the IRS in areas of the mesolimbic circuit

3 MATERIALS AND METHODS

3.1 Animals

We used 10 male Wistar rats of two months of age. The animals were purchased from Janvier Labs (Saint-Berthevin, Cedex, France) and housed in couples to reduce the stress induced by social isolation. Food and water were available *ad libitum*. They were maintained in a light / dark cycle of 12 / 12h and all behavioral experiments were carried out during the dark phase of the rats. All experimental procedures were approved by the Ethics Committee and according to the university Jaime I guidelines for the use of laboratory animals. All procedures were in line with directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes.

3.2 Conditioning apparatus

A square box (60 cmx 60 cm x 60cm) (1.2 cm thick), similar to the classical open filed, was used for the wheel training. The three-chamber device (two rooms (39cm x 29.2cm x 49cm) separated by the smaller compartment (39cm x 19cm x 49cm)

was used to establish the contexts A, and context B, both contexts had different walls and floors. Context A had white vertical lines (2.5 cm wide, 2.5 cm apart) and straight bars embossed on the floor. Context B had horizontal lines (2.5 cm wide, 2.5 cm apart) on the walls and uneven floor with embossed circles. In the experimental procedure we paired context A to the wheel (CS+) and context B to an empty cage (CS-).

3.3 Conditioning place preference paradigm

CPP protocol had duration of three weeks during which a particular context was associated (walls with vertical white lines and floor of bars or walls with horizontal lines and floor of circles) with voluntary exercise on a wheel CS + or with an empty cage CS-. Every day before starting the experiment, the rats were left to habituate to the room for thirty minutes. The test day, the rat was left to choose between contexts A or B for 10 minutes and the time spent in context A (CS+) and context B (CS-) was measured. During the conditioning phase, after habituation, on the training phase the rat was left to free access to the running wheel for an hour. After this time, the rat was confined to context A (CS+) for 30min. Following day, the same rat spent one hour in an empty box, followed by half an hour confinement to context B (CS-). This pairings were repeated 8 times (sixteen days). All contexts were cleaned with 30% ethanol. Following the condition phase for 16 days, the tests consisted of 20 minutes, ten minutes were taken as habituation + ten minutes for test. Briefly, after insulin or saline injection into the ventricle, we waited for 1 hour before started the test trials. All test trials began by placing the rat in the midline of the chamber. For the preference score we measured the number of times it is in each context (with all four legs inside the compartment). The mean time spent in both compartments was monitored with a digital video recorder Smart 2.5.19, Panlab, Barcelona, Spain. Only when all four legs were in that chamber, a criterion for considering that rat was in each compartment. Rats were returned to their boxes after each conditioning trial.

3.4 Stereotaxic surgery

Rats were anesthetized with ketamine (Imalgene 55 mg/kg i.p.; Merial Laboratories SA, Barcelona, Spain) and medetomidine (Xilagesic 20 mg/kg i.p.; Lab

Calier, Barcelona, Spain), and placed in a stereotaxic frame (David Kopf Instruments 963-A, Tujunga CA, EE.UU). The skull was exposed and a guide cannula (3,4 mm) was implanted in the right lateral cerebral ventricle. The coordinates were AP 0.48 mm, ML 0.1 mm, DV 4 mm from bregma (Paxinos & Watson 2013). Animals were used for biological techniques after a recovery period of 7 days.

3.5 Immunohistochemistry

Immediately after conditioning, rats were anesthetized with sodium pentobarbital (120mg/Kg Eutanax, Fatro, Barcelona, Spain), and were perfused with physiological saline (0.9%), followed by 4% paraformaldehyde (PF) fixative in 0.1 M phosphate buffer (PB) pH7.4. Following perfusion, brains were extracted, post-fixed overnight at 4 °C in the same fixative that was used for perfusion. Next day, the brains were immersed in 30% sucrose for 48 hours acting as cryo-protectants, frozen at -80°C, and 40 μp wide coronal sections of the brain were sliced using a Leica Microscope SM2010R (Leica Microsystems, Heidelberg, Germany). The brains were cut in the rostral to caudal direction and from each brain six sets of cuts were collected which were stored at -20 ° C for further analysis.

pERK (Phospho-p44/42 MAPK), rabbit anti-pERK (–Cell Signaling Technology-), expression was assessed by immunohistochemical analysis; diluted 1:100 was used. IRS-1 (insulin receptor substrate-1), rabbit anti-IRS1 (–Abcam, Cambridge, UK-) expression was assessed by immunohistochemical analysis; diluted 1:250 was used. IRS-2 (insulin receptor substrate-2), rabbit anti-IRS2 (-Abcam, Cambridge, UK-) expression was assessed by immunohistochemical analysis; diluted 1:300 was used. Tissue slices were rinsed twice in 0.05 M Tris-buffered saline (TBS) pH 8.0 and once in TBS with 0.2% Triton X-100 at room temperature. Then, sections were incubated in blocking solution to reduce non-specific labelling. Primer antibody diluted 1: 100, 1: 250, and 1:300 respectively, in 0.01 M phosphate buffered saline (PBS) to which standard 2% ass donkey serum was added, TBS with 0.2% Triton X-100 and serum albumin Bovine (BSA), was incubated overnight at room temperature. After, tissue slices were incubated in the 1:200, all primer antibodies biotinylated secondary antibody solution Jackson diluted in TBS with Triton X-100. Two hours later, slices were transferred to the avidin-biotin-horseradish peroxidase complex solution (Standard Elite ABC kit, Vector Laboratories) for 90 minutes, followed of two rinses in TBS with

0.2% Triton X-100, and two more in 0.05 M Tris / HCl pH 8.0. Then, slices were processed into 0.05 M Tris / HCl pH 8.0 to which 3.125 mg of DAB and 2 µl of H 2 O 2 were added over 15-20 minutes. Reaction was stopped by adding excess 0.05 M Tris / HCl pH 8.0, followed by several rinses in PBS. Finally, slices were mounted on gelatinized, air-dried, alcohol dehydrated, xylene-lined slides, and covered with the coverslip using the DPX mounting medium.

3.6 Imaging analysis

pERK, IRS-1, and IRS-2 images were acquired using a Nikon Eclipse E800 (Nikon, Tokyo, Japan), equipped with a Nikon DMX-2000 camera connected to a PC with Leica acquisition software (Leica Microsystems, Heidelberg, Germany). pERK positive neurons were quantified in all different images of bregma from NAcc, PFC, and VTA taken with the 20x objective from all groups. An observer blind to experimental conditions quantified the number of pERK positive neurons.

3.7 Statistical Analysis

Conditioned place preference data were expressed as a percentage of the time in context CS+ (time spent in the CS+/total time). pERK quantification were expressed as a mean ± SEM of each groups. Statistical significance was calculated using one way-ANOVA, followed by a Bonferroni post-test to analyze differences between treatments using Graphpad Prism version 7 software.

4 RESULTS

4.1 Intraventricular insulin injection showed no significant differences on conditioned place preference using exercise as a reward stimulus

In order to set up the behavior paradigm to study insulin/IGF1 signaling effect in preference for a rewarding stimulus such as exercise, we performed a conditioned place preference (CPP) pilot experiment (Fig.2A). In this study we analyzed the behavior of a naïve rat (RW6), two sham (RW1; RW8) and four insulin icv inoculated rats. We found that two rats that had insulin injection (RW4, RW7) had a preference score of (68,66

and 172,48 respectively) suggesting they had acquired place preference for the context associated with the running wheel. The other, two rats that had insulin injection (RW2 and RW3) did not acquired preference (-29,16 and -18,67). Neither of the rats control displayed preference for CS+ sham (-293,61 and 21,39) or naïve (-113,22) (Fig 2B). During the training phase the first latency time (time before going up the wheel the first time) was measured every day (Fig 2C). In an attempt to establish whether or not the motivation to the wheel during acquisition time could be correlated to preference later on in the test. We did not find this kind of correlation since the latency time is highly variable.

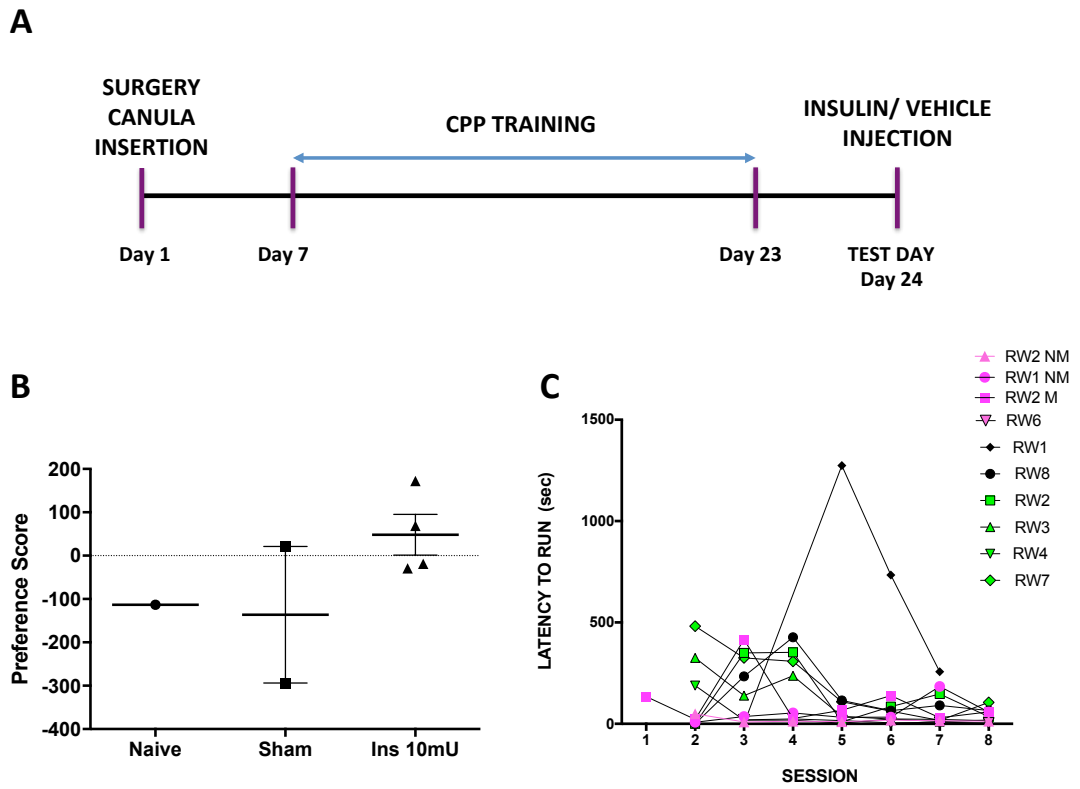

Fig.2. Intraventricular insulin injection showed no significant differences on a conditioned place preference (CPP) paradigm using exercise as a reward stimulus. *(A) CPP behavioral paradigm experimental design. Day 1 rats were subjected to surgery for cannula insertion. After one week CPP training started. CPP training consists in 8 pairings of wheel with context A (CS+) or empty box with context B (CS-). These contexts were the side rooms in a three chamber apparatus. Day 24 (test day), rats were either non injected (naïve); injected with vehicle (sham) or injected with insulin (10mU). Ten minutes after injections the rats were placed in the three chamber apparatus (10minutes test) to choose between context A (CS+) or context B (CS-). (B) Preference Score .Time in context A (CS+ associated to wheel) minus time*

in context B (CS- associated to empty box). (C) Latency . Data shows the time (seconds) before the rat stars running in the wheel each da n=2-4

4.2 pERK phosphorylation increases in specific brain regions of the insulin injected rats

Reward-related behaviors have been associated with phosphorylated ERK (pERK) levels in many areas of the dopamine reward system (Gerdjikov et al. 2004; Lu et al. 2006). On the other hand, insulin/IGF1 signaling induces ERK phosphorylation; therefore we wanted to ascertain if ERK activity could correlate with the behavior we observed in the CPP paradigm. To that end we performed ICC with an antibody detecting pERK and quantified positive cells in different in areas of the mesolimbic circuit after the insulin injection and the CPP paradigm.

Prefrontal cortex (PFC) was analyzed at bregma 3mm (Fig. 3) and Bregma 3.24 mm (Fig. 4). Representative images of sham (Fig. 3A, 4A), naïve (Fig. 3B; 4B); insulin injected with negative preference score (INS CS-)(Fig. 3C, 4C); and insulin injected with positive preference score (INS CS+)(Fig. 3D-4D). Quantification of the number of pERK positive cells in PFC (both PrL and IL), show no differences between groups (Fig 5).

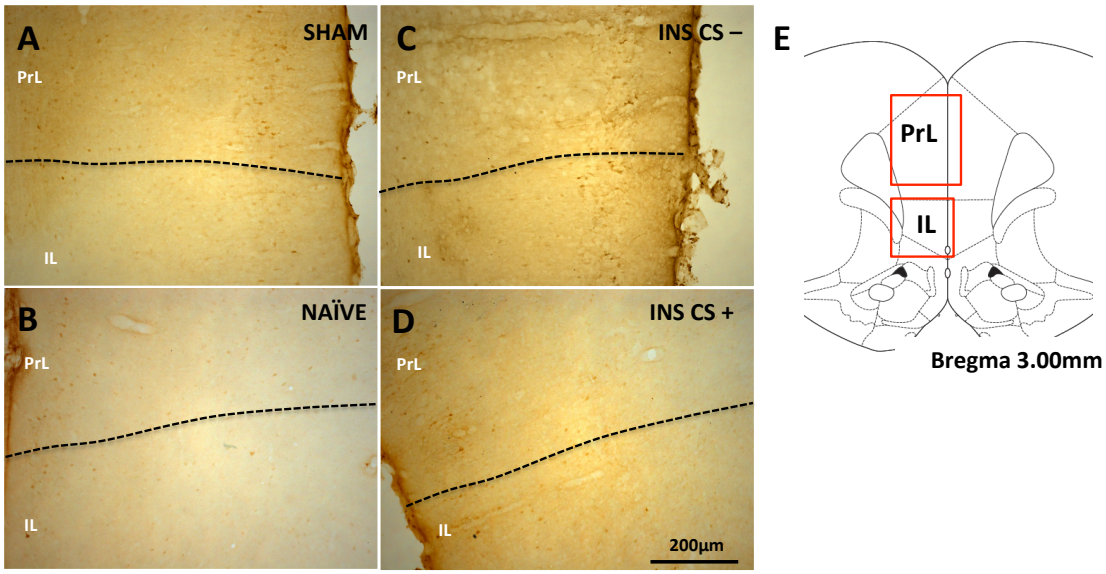

Fig.3. P-ERK labeling in Prefrontal Cortex (PFC). *Representative images of pERK staining in PFR from sham (A); naive (B), insulin injected but not conditioned, INS CS- (C) and insulin injected and conditioned, INS CS+ (D). Paxinos atlas drawing level bregma 3.00mm (E). Scale bar 200 µ m. Black dashed line DELIMITS prelimbic (PrL) and infralimbic (IL).*

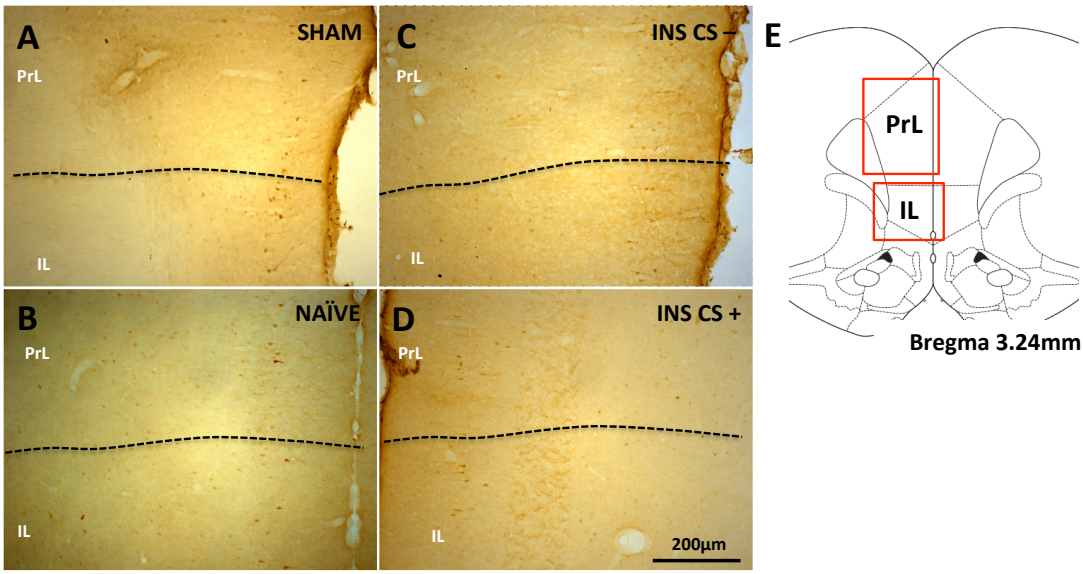

Fig.4. P-ERK labeling in Prefrontal Cortex (PFC). *Representative images of pERK staining in PFR from sham (A); naive (B), insulin injected but not conditioned, INS CS- (C) and insulin injected and conditioned, INS CS+ (D). Paxinos atlas drawing level bregma 3.24mm (E). Scale bar 200 µ m. Black dashed line DELIMITS prelimbic (PrL) and infralimbic (IL).*

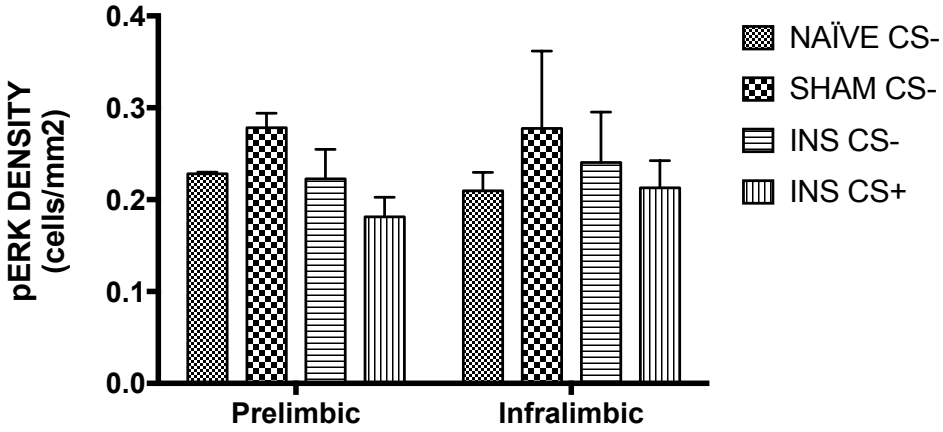

Fig 5. Cell density quantification of P-ERK positive cells in prelimbic and infralimbic. *Representative of P-ERK positive cells in different levels of bregma in prefrontal cortex from sham, naive, insulin injected but not conditioned, INS CS- and insulin injected and conditioned, INS CS+.*

Next, we quantified pERK in the nucleus accumbens core (AcbC) and shell (AcbSh) at different bregma levels. At Bregma 1.2mm representative images of pERK in sham (Fig. 6A), naïve (Fig. 6B), INS CS- (Fig. 6C), and INS CS+ (Fig. 6D) groups. Posteriorly, at bregma 1,92mm, we detected pERK in sham (Fig. 7A), naïve (Fig. 7B), INS CS- (Fig. 7C), and INS CS+ (Fig. 7D) groups. Finally we analyzed pERK at

bregma 2.28mm in sham (Fig. 8A), naïve (Fig. 8B), INS CS- (Fig. 8C), and INS CS+ (Fig. 8D) groups. Quantification of the number of pERK positive cells in NAcc at the three levels combined show that pERK in the NAcc core in the INS CS+ rats was higher (0,83 ± 0,13 SEM), than the others sham (0,43 ±0,1 SEM); naïve (0,47 ±0,12 SEM) or INS CS- (0,57 ±0,15 SEM), however, no significantly different (Fig 9).

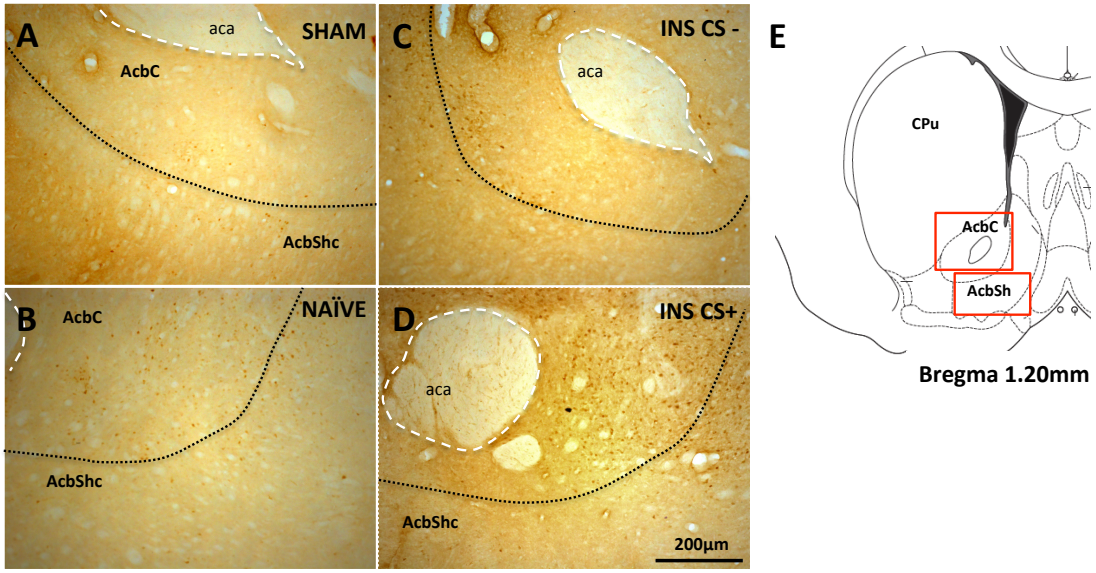

Fig.6. P-ERK labeling in Nucleus accumbens (Bregma 1.20mm). *Representative images of pERK staining in nucleus accumbens from sham (A); naive (B), insulin injected not conditioned, INS CS- (C) and insulin injected conditioned, INS CS+ (D). Black dashed line delimits nucleus accumbens CORE (AcbC) AND SHELL (AcbSh). Paxinos atlas drawing level Bregma 1.20mm (E). Scale bar 200 µ m.*

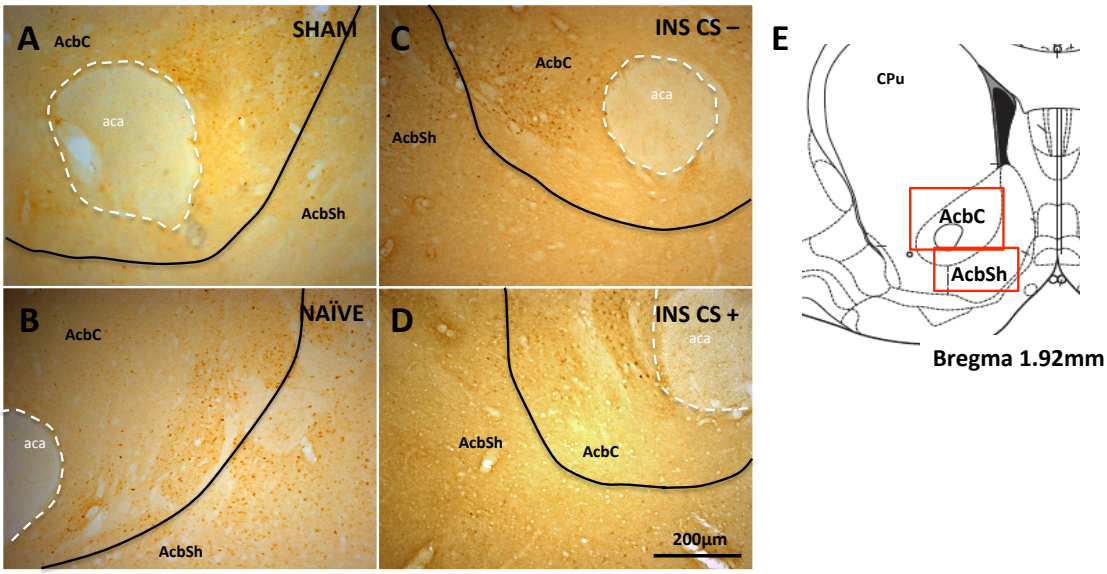

Fig.7. P-ERK labeling in Nucleus accumbens (Bregma 1.92). *Representative images of pERK staining in Nucleus accumbens from sham (A); naive (B), insulin injected but not conditioned,*

INS CS- (C) and insulin injected and conditioned, INS CS+ (D). Paxinos atlas drawing level Bregma 1.92mm (E). Scale bar 200 μm. Black dashed line delimits nucleus accumbens core (AcbC) and shell (AcbSh).

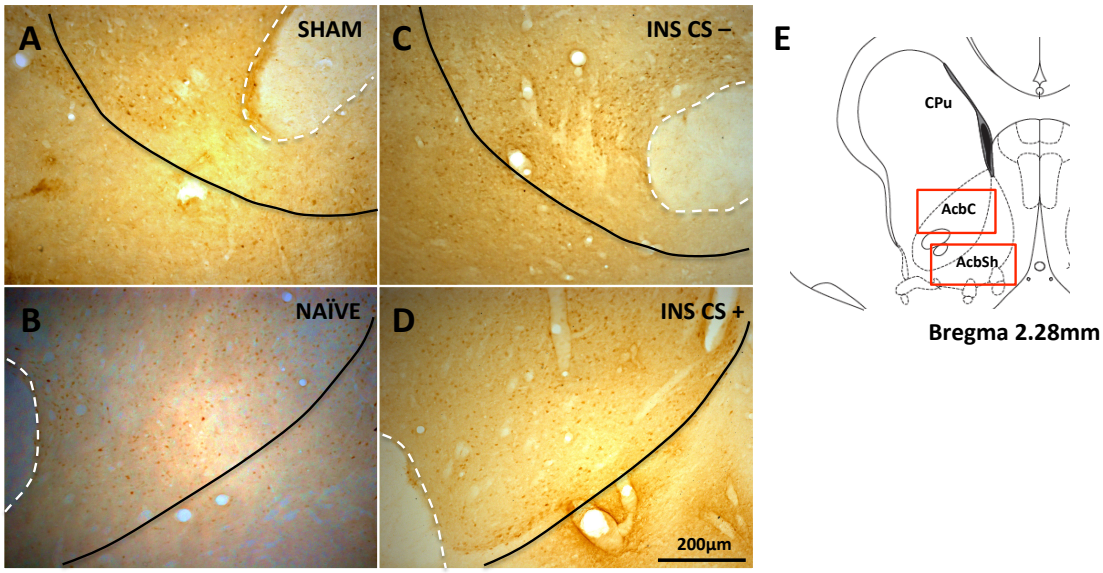

Fig.8. P-ERK labeling in Nucleus accumbens (*Bregma 2.28mm)* ***.*** *Representative images of pERK staining in Nucleus accumbens from sham (A); naive (B), insulin injected but not conditioned, INS CS- (C) and insulin injected and conditioned, INS CS+ (D). Paxinos atlas drawing level Bregma 2.28mm (E). Scale bar 200 μm. Black dashed line delimits nucleus accumbens CORE (AcbC) AND SHELL (AcbSh).*

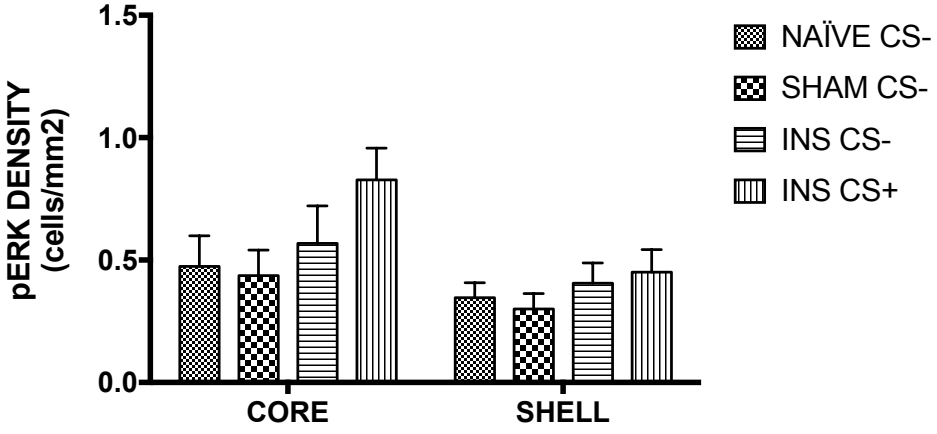

Fig 9. Cell density quantification of P-ERK positive cells in core and shell. *Quantification of P-ERK positive cells in different levels of bregma in Nucleus accumbens from sham, naive, insulin injected but not conditioned, INS CS- , and insulin injected and conditioned, INS CS+.*

Finally, we analyzed pERK at the ventral tegmental area (bregma -6.72mm). Representative images of sham (Fig. 10A), naïve (Fig. 10B), INS CS- (Fig.10C), and INS CS+ (Fig 10D) groups are shown. Quantification shows a clear increase in pERK positive cells in the INS CS+ group (35,55±SEM?) compare to sham (0,31 ±), naïve (0,26 ±) and INS CS- (0,28±n) subjects. However statistical difference was not reached (Fig. 10E).

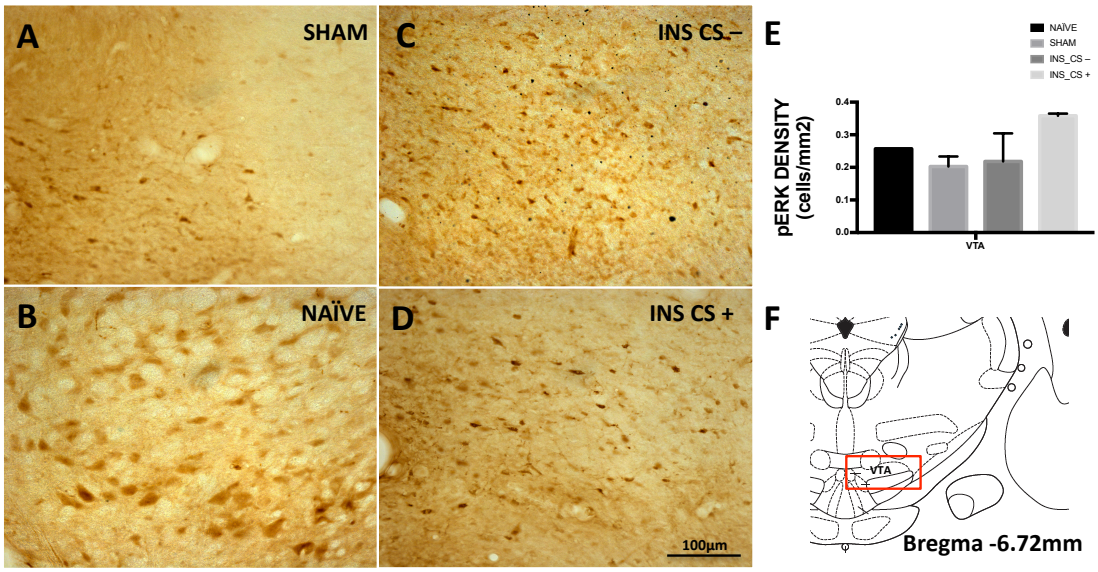

Fig.10. P-ERK labeling in Ventral Tegmental Area (VTA). *Representative images of pERK staining in VTA from sham (A); naive (B), insulin injected but not conditioned, INS CS- (C) and insulin injected and conditioned, INS CS+ (D). Cell density quantification of P-ERK positive cells in VTA (E). Paxinos atlas drawing level Bregma -6.72mm (F). Scale bar 100 µ m.*

4.3 IRS1 and IRS2 expression displays a different pattern in prefrontal cortex nucleus accumbens and ventral tegmental area

To further the study of insulin/IGF1 signaling in the CPP to exercise, we aim to locate the insulin/IGF main intracellular mediators, IRS2 and IRS1, in the brain regions of the mesocorticolimbic system. Naïve rats were used to analyze IRS1 and IRS2 expression. In PFC we observed IRS2 very abundantly expressed a throughout the prelimbic (PrL) (Fig. 11A) whereas IRS1 was sparse (Fig. 11B). IRS2 displays a similar pattern of expression in the infralimbic (IL) areas Fig. 11C), whereas IRS1 seems concentrated in a reduced area within (Fig 11D).

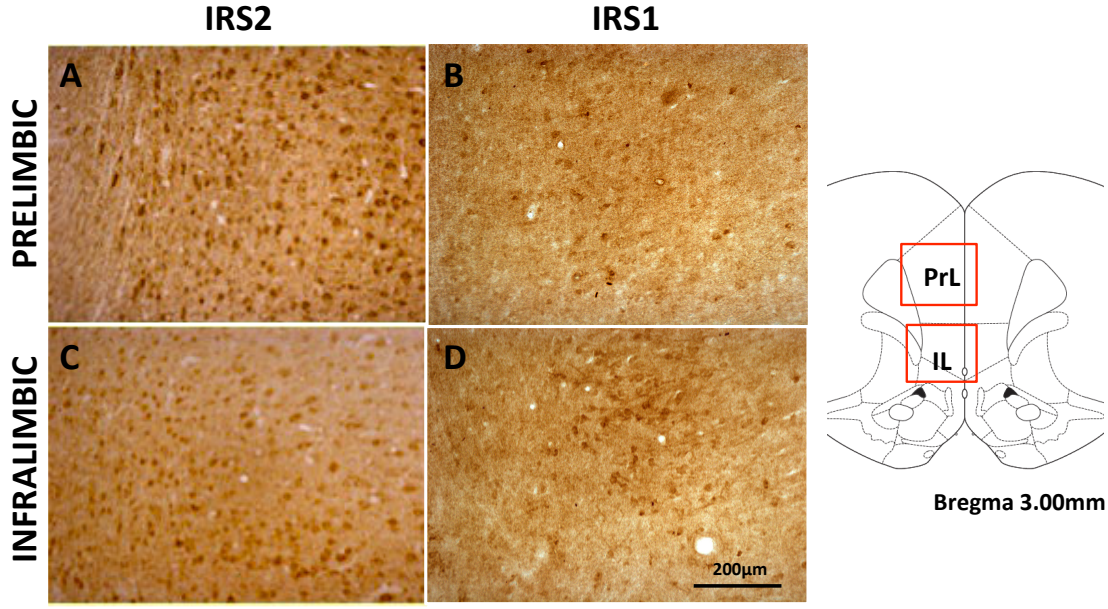

Fig. 11. IRS2 and IRS1 Staining in Prefrontal Cortex. *IRS2 (A), and IRS1 (B) labeling in prelimbic region. The IRS2 (C), and IRS1 (D) staining in the infralimbic region. Paxinos atlas drawing level Bregma 3.00mm (F). Scale bar 200 μ m.*

In NAcc IRS2 labeled cells were abundant and spread throughout the area without a definite pattern of expression; in NAcc shell we observed IRS2 labeled cells abundant, whereas sparse in the NAcc core were (Fig. 12A). However, IRS1 positive cells were less abundant, for both NAcc shell and core, with a similar pattern between them (Fig. 12B). Finally, at the VTA we observed IRS2 abundant labeling (Fig.12 C) whereas IRS1 labeled cells appear sparse. No definite pattern of expression was observed (Fig.12D).

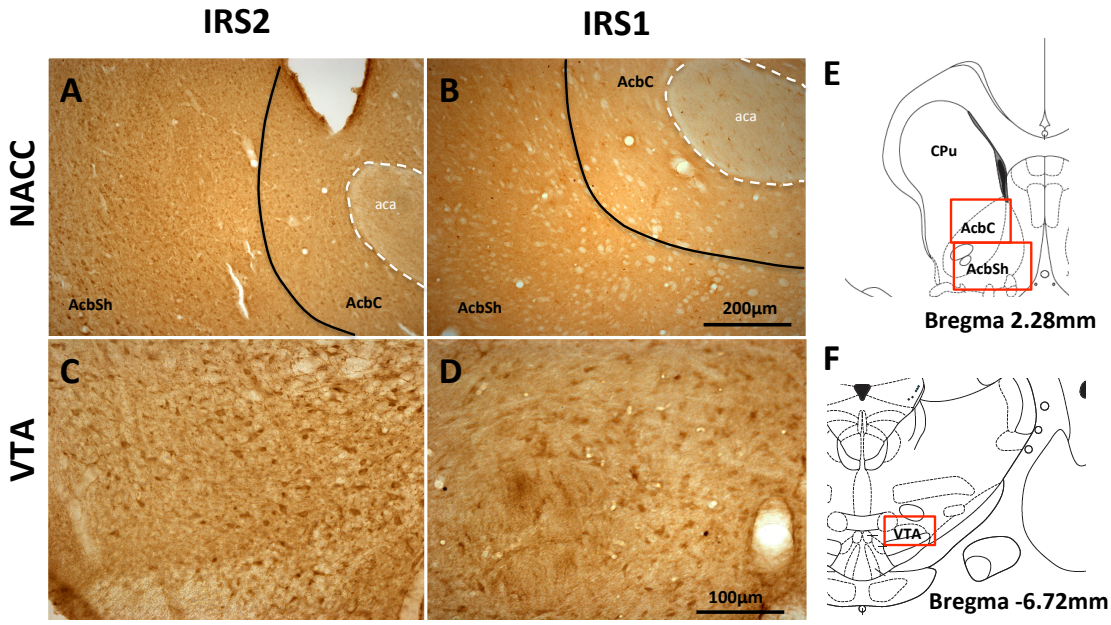

Fig. 12. IRS2 and IRS1 Staining in Nucleus Accumbens (Nacc), Ventral Tegmental Area (VTA). *IRS2 (A) and IRS1 (B) labeling in Nacc. Analysis of the VTA show IRS2 (C) and IRS1 (D) staining . Paxinos atlas drawing level bregma 2.28mm (E); -6.72mm (F). Image A, B* scale bar *200 µ m, and C,D* scale bar *100 µ m.*

5 DISCUSSION

Growing evidence has shown that insulin has a wide range of molecular targets in the central nervous system (CNS). Studies suggest that insulin is implicated in a multitude of brain functions, such as learning, memory, cognitive function, appetite, and others. If this is so, it is logical to think that failures in insulin signaling can lead to different types of brain disorders. Until the last years it was believed that insulin only entered the CNS through a blood-brain barrier (BBB), but there are studies that suggest the possibility of endogenous production, however, has not yet been sufficiently investigated. So, further studies are clearly needed to better understand insulin transport into the brain in health and disease (Gray et al. 2014; Heni et al. 2015). Evidence at the last decades, have found a role of the insulin in food intake, plasma glucose levels and brain pathways associated with reward (Greenwood et al. 2011; Labouebe et al. 2013; Garcia et al. 2005; Galici et al. 2003; Figlewicz et al. 2006; 2008; Davis et al. 2010). Regarding to reward-related behaviors, Labouèbe et al., 2013 reported data about the insulin in the VTA inhibited food anticipatory behavior and CPP for food, suggesting

that insulin may attenuate the salience of food-related contexts or cues (Labouebe et al. 2013). In addition, Figlewicz and collaborates contribute in this subject, they suggested that effects of insulin on the mesolimbic dopamine system can interact with limbic system to diminish the reward value of stimuli ((Figlewicz 2003; 2006; 2008). Insulin can interact to the actions drugs into the CNS; there is converging evidence that this occurs through insulin regulation of the dopamine (DA) transporter (DAT), such as AMPH or methamphetamine (METH) psychostimulants drugs, which related DA neurotransmission. DAT activity decreases in rats with low blood insulin levels (Samandari et al. 2013; Owens et al. 2005; Galici et al. 2003; Sevak et al. 2009). Moreover, this modulation of insulin into the brain can developed a long-term plastic changes at the molecular level, such as a DAT function (Owens et al. 2005). Some authors demonstrated that insulin can presynaptically enhance the function of cocaine sensitive DAT into the nucleus accumbens, and may decrease impulsive behaviors (Schoffelmeer et al. 2011). Taken together, these data, offer some highlights to understand insulin-signaling effects into, mainly, the mesolimbic dopamine system and their adjacent regions that involved the reward-related behaviors.

In the present study, we have tested the CPP paradigm to study the effect that insulin signaling exerts on the reward system. To that end insulin intracranial injections have been tested in a paradigm of CPP with voluntary exercise. The assumptions is that voluntary physical activity can act as a positive reinforcer stimulus (Greenwood et al. 2011; Lett et al. 2001; Lett et al. 2000). In addition, previous data in our laboratory with three naïve animals showed that two of them acquired preference for the context paired to the running wheel (data not shown).

In the CPP paradigm, we observed that two rats of the four rats that had insulin injection had acquired place preference for the context associated with the running wheel, whereas naïve and sham groups did not. Although it is a high variable paradigm, this data may suggest that insulin could have a positive effect in the behavioral paradigm. In addition, we could not rule out the effect of insulin improving spatial memory, which would also be implicated in the association of context to the pleasant effects after voluntary exercise. Some studies suggested that insulin-like growth factor (IGF-1), as well as other neurotrophic factors, may be mediator for the exercise-derived hippocampal plasticity (Cotman et al. 2007; Carro et al. 2001); other authors involved

IGF-1 as mediator to physical exercise-enhanced hippocampal neurogenesis, they showed that continued running reversed the suppression of hippocampal cell proliferation and impaired spatial learning by CORT treatment (Yau et al. 2012). Moreover, the prevention of memory loss and structural changes induced by streptozotocin can be reverted by insulin (Bahramian et al. 2016), they shown that there is a dose dependency in the protective effect of insulin (Ghasemi et al. 2014; Bahramian et al. 2016).

ERK has been shown to mediate a number of neuronal processes, including reward effects. Since ERK has relevance in insulin/IGF1 signaling, and we also know that ERK is implicated in the learning that is expressed in NAcc, is necessary try to elucidate whether these effects can be extrapolated to the wide variety of reinforcers. Previous studies supported our assumption, if both glutamate and dopamine are upstream regulators of ERK to both natural (Shiflett et al. 2008), and drug (Lu et al. 2006; Zamora-Martinez & Edwards 2014; Edwards et al. 2011) rewards, could be pERK a suitable marker to test the preference of animals to running wheel. Some studies indicate that ERK activity in the accumbens (and possibly dorsal striatum) probably serves two distinct roles: 1) during CPP training, accumbens ERK activity mediates consolidation of learned associations between the unconditioned rewarding effects of the drug and the drug-paired context; 2) during CPP testing, accumbens ERK activity mediates acute expression of cocaine-cue-conditioned responses (Gerdjikov et al. 2004; Valjent et al. 2004; Girault et al. 2007; Lu et al. 2006). All drugs of abuse increased ERK phosphorylation in several brain regions through to the action of the DA. Some these regions are NAcc or PFC, which belong to mesocorticolimbic system. Also, non-addictive drugs can activate ERK; however,. Valjent and collaborators have found ERK activation by psychoactive drugs, and this activation was inhibited by a dopamine receptor (DR) D1 antagonist in the striatum. In addition, they demonstrated that systemic inhibition of the ERK pathway prevents (CPP) induced by cocaine or other psychoactive drugs. (Girault et al. 2007; Valjent et al. 2004; Lu et al. 2006; Koya et al. 2009b; Gerdjikov et al. 2004). According to these data, it is likely that similar effects can be observed in experiments where the contexts in the CPP paradigm is paired to voluntary physical exercise (using a running wheel) as natural reinforcer stimulus

We have found quantification higher number of pERK positive cells in a rats that had been subjected to icv insulin injection and that had acquired place preference (INS CS+) group in a some specific areas nucleus accumbens (NAcc) core and ventral tegmental area (VTA). Interestingly, insulin injected animals that had not acquired place preference (INS CS-), showed a pERK labeling similar to sham and naïve groups. In other regions of the mesocorticolimbic system such as prefrontal cortex (PFC) or NAcc shell we found no differences between groups. This could suggest that the responses of animals in a CPP paradigm can be improved by insulin, due to insulin signaling into specific reward areas, but not all. Moreover, others studies suggest that VTA injections of insulin block the impact of food-related contextual stimuli. Insulin-induced reduction in activity during anticipation of food may reflect changes in simple appetitive behaviors displayed routinely prior to consumption of a meal; an effect that is mediated by the salience of food-related cues. (Labouebe et al. 2013), whereas other studies that reported a role in regulation of insulin in the VTA. This hormone is capable of signaling within brain reward circuitry, and perturbations of their signaling mechanisms in these regions decrease feeding behavior and consumption of palatable foods (Davis et al. 2010; Pardini et al. 2006b). . However, we need to test the hypothesis with further experiments to understand the role of insulin modulating reward-related behaviors.

Since insulin action is mediated by the insulin receptor substrates (IRS) we set out to analyzed the expression pattern of these proteins, in particular IRS1 and IRS2. We have observed that IRS2 is expressed in prelimbic (PrL) and infralimbic (IL) region of PFC; however, IRS1 labeled cells appear sparse. We found that similar pattern in the NAcc, the number of IRS2 labeled cells was higher than IRS1, that was expressed in few cells. Also in the VTA IRS2 expression was higher than IRS1.

The conclusion of our study is that, given the abundance of IRS2 in all these regions maybe IRS2 the substrate that mediates insulin effects on the reward mechanisms. In any case, we cannot out the effect of IRS1. Further studies on the phenotype of these IRS expression neurons will be necessary to understand the role of these proteins mediating insulin/IGF1 effects.

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