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TITOLO DELLA TESI DI DOTTORATO

RECONSOLIDATION OF APPETITIVE INSTRUMENTAL MEMORY AND THE METAPLASTIC EFFECTS OF NMDA RECEPTORS BLOCKADE IN RATS

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Coordinatore: Chiar.mo Prof. Leonardo Chelazzi

Firma _____

Tutor: Chiar.mo Prof. Cristiano Chiamulera

Firma _____

Dottorando: Dott. Alessandro Piva

Firma _____

Abstract

Obesity is one of the leading cause of death in world's population, due to the comorbidity with pathologies like cardiovascular or mental disorders. Deviating from its role as a primary need, obese and overweight people tend to use food as a source of pleasure or to relieve from anxiety, typical of addiction to drugs. Thus, due to the commonality with drug abuse, like the impulsive-to-compulsive progression or the neurotransmitters release, overconsumption of high palatable food has been described as a form of addiction. As initially done for fear and drug maladaptive memories, food addiction has been investigated with memory reconsolidation studies. Reconsolidation theory stated that after stabilization, memory can return into a vulnerable state with re-exposure to stimuli previously associated to the initial learning and, once reactivated and destabilized, it needs reconsolidation to be restabilized. During the vulnerable phase, memory can strengthened or disrupted, and memory disruption could prevent relapse. The processes of memory destabilization and restabilization have been shown to depend on glutamatergic signaling. Moreover, glutamate receptor activity has been proposed as modulated by metaplasticity, a novel concept according to which neural stimulation could influence future synaptic activity even after disappearing of the acute effect. Thus, the goals of the present project was to investigate i) whether sucrose instrumental memory can undergo reconsolidation and ii) if it is possible to block reconsolidation with the NMDARs antagonist MK-801 given under a metaplasticity protocol.

After 10 days of training to sucrose self-administration, rats were fasted for 14 days and finally treated with vehicle or MK-801 24 hours before retrieval. Then, rats were exposed to memory retrieval (Ret) or no-retrieval (No-Ret) and, 24 hours later, a reinstatement test evaluated sucrose seeking behaviour. Separate groups of rats were sacrificed 2 hours after memory Ret/No-Ret for the molecular analyses of Zif268, rpS6P and glutamate receptors levels in memory and reward key brain areas: nucleus accumbens (NAc) and amygdala (Amy).

Results showed that sucrose instrumental memory undergo reconsolidation, as indicated by the increase of Zif268 and rpS6P in NAc and Amy. These results were further supported by the increased level of GluN2B (destabilization marker)

and GluA1 (reactivation marker) in Amy, leading us to propose the Zif268/rpS6P two-component molecular assay as a valid and reliable method for the assessment of instrumental memory reactivation and reconsolidation. Furthermore, we demonstrated that the metaplastic treatment with MK-801 significantly decreased instrumental responding at the behavioural test only when administrated 24 hours before Ret, and this inhibition was associated to a significant decrease of Zif268 in NAc shell and of rpS6P in central Amy. Moreover, acute MK-801 significantly increased GluA1, GluN2B and mGluR5 in NAc and GluN2B in Amy. Here we demonstrated that NMDARs blockade affected sucrose instrumental memory, hypothesizing that the metaplasticity effect of MK-801 could have induced a switch from reconsolidation-to-extinction occurrence. However, it remains to be clarified the molecular mechanisms allowing for instrumental responding inhibition even at time MK-801 has been completely eliminated, and whether this inhibition is long-lasting, making it a possible therapeutic treatment for relapse on food addiction.

CONTENTS

1. INTRODUCTION

Drug addiction, or substance use disorder as recently redefined in DSM-V, is a chronic relapsing disorder characterized by drug seeking and taking compulsivity, weak control over drug self-administration, impaired social function and emergence of negative emotional state, such as anxiety, dysphoria and/or aggressiveness (American Psychiatric Association, 2013; Koob and Le Moal, 1997). As other chronic diseases, also substance use disorder is characterized by susceptibility due to genetic or environmental risk factors, as well as to age, stress, physical injury or traumatic emotional experiences (Kendler et al., 2003; 2008). The view of addiction as a medical condition provided support for the integration of substance use disorder treatments in the healthcare systems, and resulted in the development of medicine and clinical trials addressed to find new therapeutic strategies for addiction (Volkow et al., 2016). In Europe, in 2015, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA, 2017) reported that 23.5 million adults between 15 and 64 years old used cannabis in the last year, 3.5 million used cocaine, 2.7 million used MDMA and 1.8 million used amphetamines. Moreover, Europe has the highest prevalence of tobacco smoking (28%) and of alcohol consumption (70%) among adults in the world (WHO, 2014).

1.1 The neurobiology of addiction

All drugs of abuse have remarkable effects into the brain, accounting for the pleasure and reward feelings usually experienced during the first uses. These sensations are those who drive individuals to use substances repeatedly, even in the case of negative consequences. The sustained drug intake can in turn induce neuroadaptation, the process underlying structural rewiring and neurochemical modulation in response to drugs. This pattern of alteration finally results in a self-sustaining loop that precipitates drug intake from a goal-directed behaviour to a compulsive-like habit, strengthening and enhancing the maladaptive condition of substance abuse (Everitt and Robbins, 2005). Thus, one of the mail goals of drug addiction research is to comprehend the neurobiological mechanisms behind this transition and the underlying alteration in brain structures and functions,

integrating animal model studies of acute and chronic effects of substances with human evidence of drug-induced changes from brain-imaging techniques (Koob and Volkow, 2016).

Information inside the human brain are processed by many different regions connecting each other to form dynamic networks responsible for distinct functions, such as learning and memory, language, reward, emotions and movements. As these functions, also addiction is based on many different brain regions; however, it relies mostly on three areas:

1) prefrontal cortex, located in the most frontal part of the brain;
2) basal ganglia, i.e. dorsal striatum (or caudate putamen) and ventral striatum (or nucleus accumbens), a group of subcortical nuclei located at the bases of the forebrain;
3) amygdala, an almond-shaped group of nuclei located beneath the basal ganglia, within the temporal lobes (Fowler et al., 2007).

These brain regions, and the regulation of reward and emotions, evolved in human and other animals to adapt to challenging environment (Figure 1). For example, reward motivated individuals to consuming food or having sex, activities controlled by the basal ganglia and essential for the survival of the species. Instead, the "fight or flight" response controlled by the amygdala evolved to face fear or dangerous situations. Prefrontal cortex is the higher order area controlling on these low-level, but fundamental, processes. Drugs of abuse take advantage of the same survival systems to establish addiction (Saah, 2005).

Despite being a part of the mesocorticolimbic pathway, prefrontal cortex, basal ganglia and amygdala own specific roles involved both in normal life situations and in the emerging of addiction:

1) prefrontal cortex is responsible for the executive functions, complex cognitive processes involved in the organization of thoughts and activities, decision making, integration of emotional and cognitive information (Barbas and García-Cabezas, 2017);
2) caudate-putamen is involved in more cognitive and motor functions, and it has been identified as the core of both goal and habitual-directed behaviour (Belin et al., 2009); nucleus accumbens has been suggested as

the interface between emotions, motivations and actions due to the inputs coming from amygdala, orbitofrontal cortex and hippocampus (Belin et al., 2009);

3) amygdala is involved in the perception of emotions such as fear, anxiety and sadness, as well as in the control of aggressiveness. In facts, it is the most activated area during abrupt stressing situations, requiring a "fight or flight" response. Moreover, amygdala plays a role also in natural rewarding activity such as food intake or sexual activity, and in the storage of emotionally relevant memories that can be helpful in dealing with future similar situations (Adolphs, 2013).

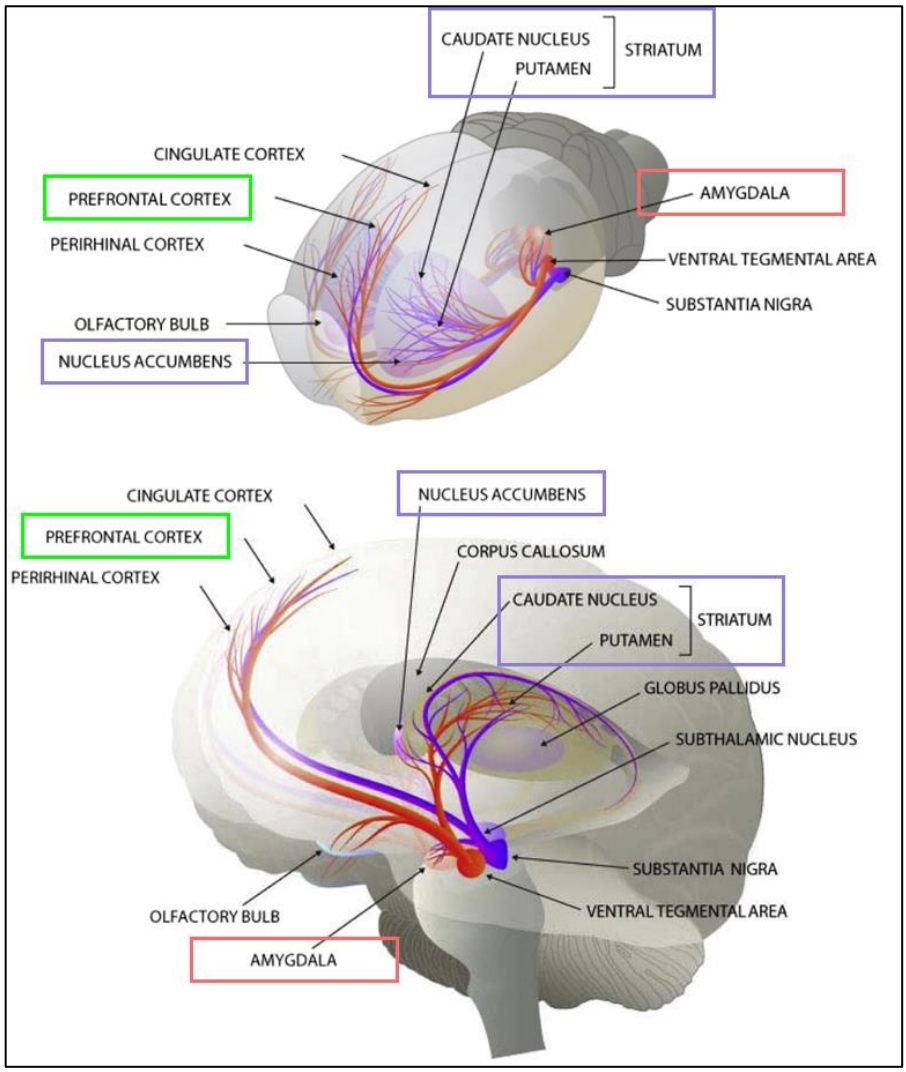

Figure 1: The homology between midbrain dopaminergic system in human and rats. (Top) The rat dopaminergic (DA) system: the DA neurons affected by

addiction are labeled in red, with cell bodies located in the ventral tegmental area and innervating the prefrontal cortex (green square), nucleus accumbens and caudate-putamen (blue square) and amygdala (red square). (bottom) DA circuitry is well conserved in the human brain, with the addiction-affected areas squared as on top. (top/bottom) In purple are represented the DA neurons of the substantia nigra and involved in other pathologies, like Parkinson's disease (PD). Taken and adapted from Kramer and Liss, 2015.

1.2 The addiction cycle

Drug-taking behaviour progresses from impulsivity to compulsivity (Figure 2) following a three-stage cycle, with each stage associated to one of the three main areas described above:

1) binge/intoxication: mostly associated to basal ganglia, it is the stage during which people experience the rewarding effects of a substance;
2) withdrawal/negative affect: mostly associated to amygdala, it is the stage during which addicted abstain from the drug, feeling negative emotions such as agitation, anxiety and malaise;
3) preoccupation/anticipation: mostly associated to prefrontal cortex, it is the stage during which addicted crave for substance after a period of abstinence (Figure 3) (Koob and Le Moal, 1997).

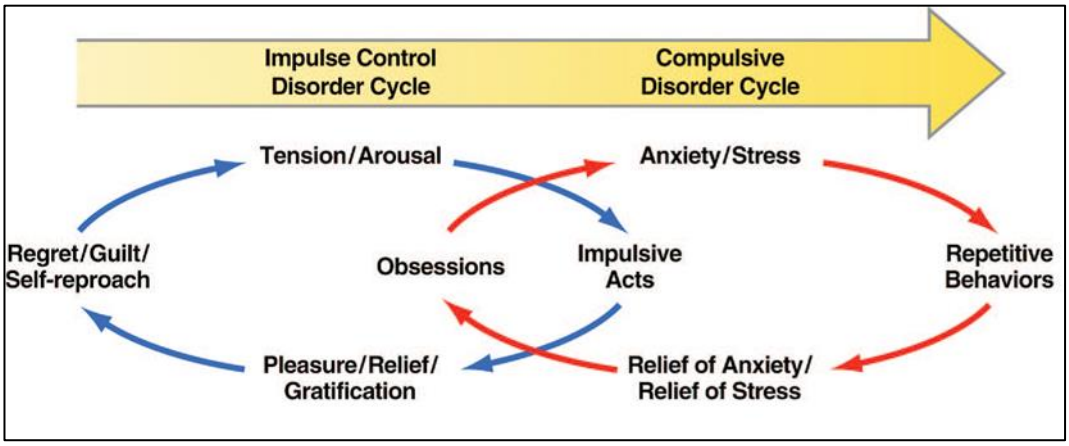

Figure 2: The progression from impulsive to compulsive behaviour. After an initial episode of arousal or stress, individuals experience an impulsive act

providing pleasure or relief. However, the impulsive act could result in a feeling of regret after relief, laying the basis for a new episode of stress followed again by impulsive act and relief. After a prolonged exposure to this cycle, impulsive acts can shift into obsession, generating an anxiety state to which only a repetitive behaviour could provide relief. However, relief is only temporary, and individuals end eventually in obsession relapse. This sequence underlie the transition from response-outcome goal-directed behaviour to the stimulus-response habit-based behaviour, typical of addictive disorders. Taken and adapted from Koob, 2011.

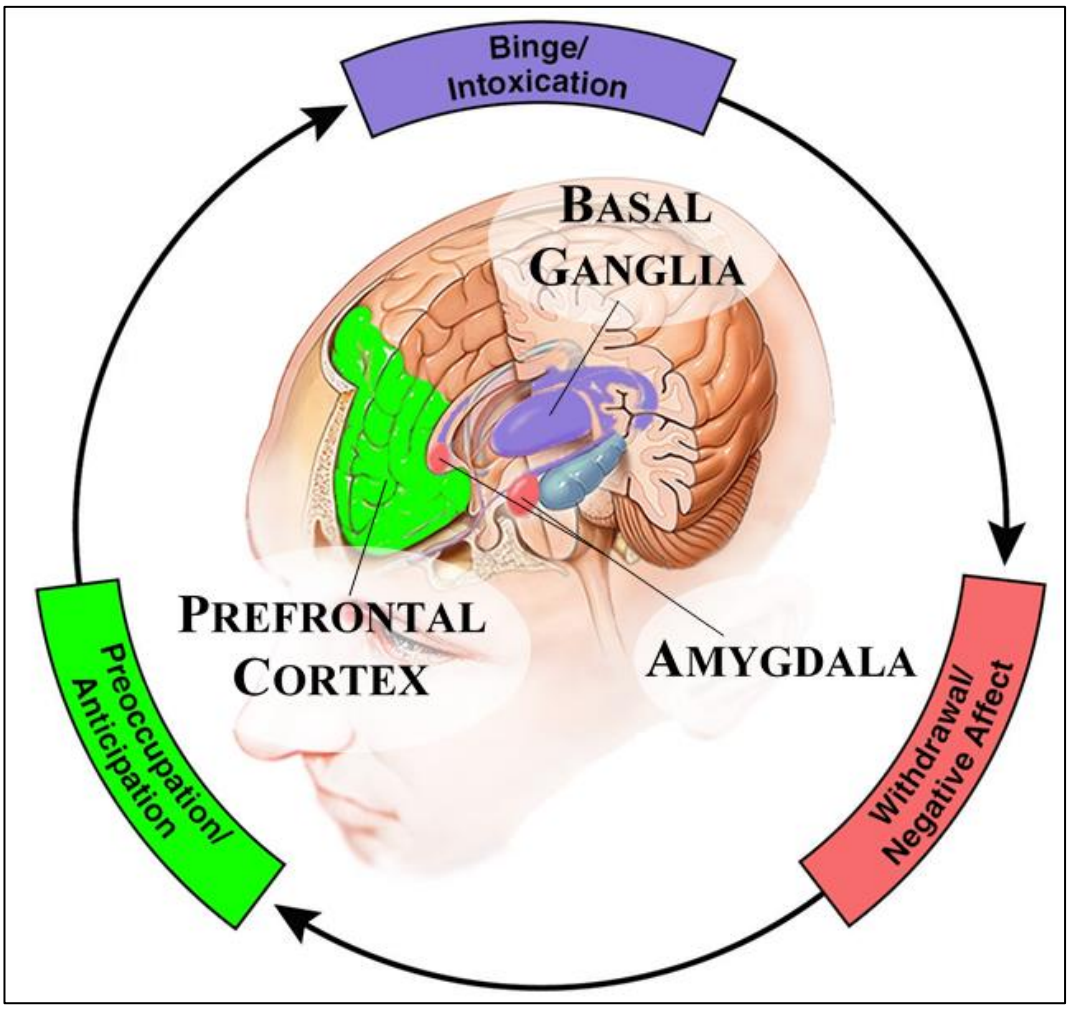

Figure 3: The three brain regions associated to the three addiction stages. In blue, there are the basal ganglia, associated to the first binge/intoxication stage; in red the amygdala, linked to the second withdrawal/negative affect stage, and finally, in green, the prefrontal cortex, base of the third preoccupation/anticipation stage. Taken and adapted from Facing Addiction in America: The Surgeon General's

Report on Alcohol, Drugs, and Health (SAMHSA, 2016) and Dr. Levent Efe Medical Illustration Studios.

1.2.1 Stage 1: binge/intoxication

The intake of addictive substances produces pleasurable effects and reward feelings, which in turn sustain the likelihood of further consumptions. This sequence is hypothesized to depend on: i) DA release in the nucleus accumbens for psychostimulants such as cocaine, amphetamine and nicotine; ii) on opioid receptors activation in accumbens (DA-independent) and in the ventral tegmental area (DA-dependent) for opiates and cannabinoids; iii) on GABAergic system activation in the nucleus accumbens and amygdala for alcohol (Koob, 2011) (Figure 4). Nucleus accumbens, the most important brain area in binge/intoxication stage is in a strategic location inside the brain, receiving limbic afferents from amygdala, frontal cortex, hippocampus and conveying information that can became motivational outputs through extrapyramidal motor cortex (Koob, 2011). The dorsal part of the basal ganglia, i.e. the dorsal striatum, does not appear to contribute to acute reinforcing effects of drug of abuse. Conversely, dorsal striatum plays a crucial role in the transition from impulsive to compulsive drug use (Everitt et al., 2008).

Besides drug abused, also stimuli that can be associated to drug intake such as people, places and even personal feelings can trigger DA release in the basal ganglia and induce craving, seeking and eventually drug taking. This motivational appeal induce by non-drug stimuli exposure is called “incentive salience”, and it has been demonstrated both in animal models and humans. The association of a drug – the unconditioned stimulus, US – to a neutral stimulus such as a light or a tone, transfer the reinforcing properties of the drug to the previously neutral stimulus, turning it into a conditioned stimulus (CS). The conditioned reinforcing properties of stimuli associated with a variety of substances, such as cocaine, heroin and even sucrose have been reported to be essential for the persistence of addictive behaviour in rats (Di Ciano and Everitt, 2004). In a report of 1997, researchers trained naïve monkeys to self-administer fruit juice as US with a

concomitant presentation of a light as CS. Surprisingly, after several days of training, electrophysiological recording of DA-transmitting neurons activity revealed that DA neurons stopped to respond to the US, while continuing to respond to the CS. This evidence highlighted the appetitive behavioural reaction transfer from the primary stimulus, i.e. the fruit juice, to its associated stimuli (Schultz et al., 1997).

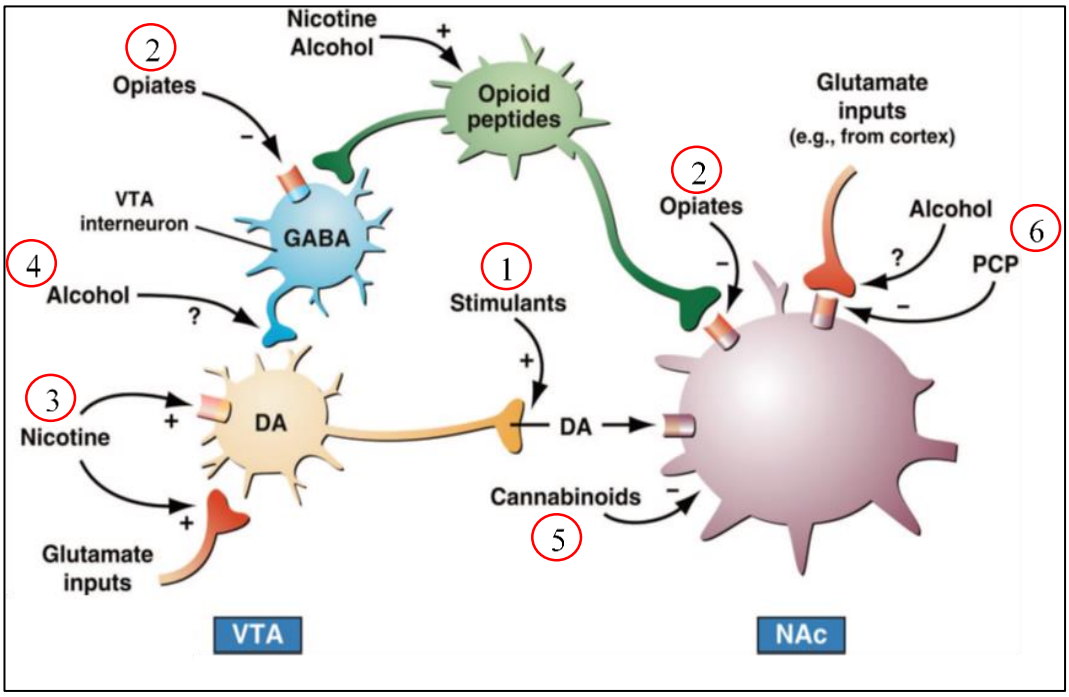

Figure 4: The common pathways of addictive substances. All drug of abuse, act directly or indirectly on nucleus accumbens (NAc) and ventral tegmental area (VTA) regions of the brain. 1) Stimulants directly increase DA release in the NAc. 2) Opiates act directly on the opioid receptors of NAc, and indirectly on the GABAergic interneuron of VTA, inhibiting them and in turn freeing up the activity of DA neurons. 3) Nicotine seems to act directly on nicotinic acetylcholine receptors (nACh) receptors on DA neurons or indirectly on glutamate inputs to DA neurons. 4) Alcohol seems to inhibit GABAergic interneurons, disinhibiting DA neurons. 5) Cannabinoids activity appears to involve CB1 receptors in the NAc neurons. Finally, 6) PCP and others NMDARs blockers like MK-801 or ketamine, antagonize NMDARs in the NAc. Taken and adapted from Koob, 2011.

1.2.2 Stage 2: withdrawal/negative affects

After binge/intoxication phase, drug abusers typically experience a period of withdrawal, characterized by negative symptoms. These, in turn, provide the basis for a future relapse on drug bingeing, considering that, normally, addicted relapse on drug taking in order to relieve from negative symptoms (Substance Abuse and Mental Health Services Administration, 2016). Symptoms of withdrawal can be both emotional, such as anxiety or stress, and physical, such as tremors, nausea and even temperature gaps (WHO, 2009). All drugs of abuse can induce withdrawal, and the severity of the effects depend on the type of substance, the duration and the intake amount. These negative affects are probably related to two distinct reasons: the first is the DA depletion caused by damping of reward circuitry in basal ganglia, while the second is the activation of the stress system based on amygdala.

In humans, DA depletion in the striatum is evident during withdrawal from cocaine (Volkow et al., 2014), nicotine (McLaughlin et al., 2015), alcohol (Stobart-Gallagher and Gomez, 2017), opioids (Kosten and George, 2002) and abused natural rewards such as food (Parylak et al., 2011) and sex (Kafka, 2010). This downregulation of DA system and subsequent reward could underlie, at least in part, the compulsive escalation to abuse, in order to obtain the initial pleasure sensations by increasing the amount of intake and consequently, increasing DA release (Koob and Le Moal, 2001).

While the striatum regulates reward feelings, the amygdala controls the stress system through the activation of stress neurotransmitters like norepinephrine, dynorphin and corticotropin-releasing factor (CRF) (Koob and Le Moal, 2008). Alcohol addiction and withdrawal, for example, induces a significant increase of stress hormones level in plasma and cerebrospinal fluid both in humans (Becker, 2012) and in rats (Patterson-Buckendahl et al., 2005). Mifepristone, a glucocorticoid receptors antagonist, was demonstrated to reduce alcohol self-administration in alcohol-dependent rats when administrated systemically or directly in the central nucleus of amygdala. Moreover, it is effective also in humans (Vendruscolo et al., 2015). Furthermore, the blockage of the dynorphin-k opioid receptor (KOR) system has been proposed as therapeutic treatment for

psychostimulant abuse (Whitfield et al., 2015). Finally, also dysregulated endogenous cannabinoid (eCB) signaling seems to contribute to the development and maintenance of addictions, including eating disorders, through increased stress responsivity and negative emotional states, thus driving to relapse (Parsons and Hurd, 2015).

1.2.3 Stage 3: preoccupation/anticipation

After abstinence, that in heavily abusers can last even few hours, addicts frequently begin to feel the need of drug intake again, an event called "craving". Prefrontal cortex, the region controlling executive functions, i.e. the ability to organize thoughts and activities, prioritize tasks, manage time and making decisions (Koob et al., 2014) as well as to regulate actions, emotions and impulses, is the main brain area involved in this phase (Substance Abuse and Mental Health Services Administration, 2016). Among the other, decision-making and action control are the most compromised functions in addicted behaviour. To frame prefrontal cortex activity in a complex scenario like addiction, scientists since early '90 divided this region in two opposed systems: the "GO circuitry" and the "STOP circuitry" (Gabriel, 1991).

The GO circuitry involves anterior cingulate cortex and the dorsolateral prefrontal cortex, with the former facilitating maintenance and selection of high attention-requiring responses, planning, initiation and monitoring of goal-directed behaviour, and the former involved in working memory, planning and strategy of actions. Incentive salience-induced drug seeking, based on goal-directed behaviour, activates glutamatergic transmission in the prefrontal cortex that in turn stimulates nucleus accumbens to release glutamate. Accumbal activation promotes incentive salience, creating an excitatory loop contributing to relapse on drug taking behaviour. Moreover, the Go circuitry contributes also to impulsive behaviours underlying drug seeking and taking through the enrolment of glutamatergic habit systems in the dorsal striatum (Koob and Volkow, 2010). Furthermore, dysregulation of the Go circuitry can reduced prefrontal cortical regulation over negative emotional salience, as reported for cocaine addicted

(Crunelle et al., 2015) and patients suffering post-traumatic stress disorder (PTSD) (Etkin and Wager, 2007).

The Stop circuitry instead involves ventrolateral prefrontal cortex and orbitofrontal cortex. The former is involved in response inhibition, sustained attention and memory reactivation, while the latter regulates the assignment of value, as well as the integration of reward and punishment (Koob et al., 2014). Stop circuitry counteracts the Go circuitry action on ventral and dorsal striatum, thus inhibiting incentive salience and impulsivity-driven drug intake (Substance Abuse and Mental Health Services Administration, 2016). In 2007, Volkow and colleagues, studying the cortical control over DA release in response to the psychostimulant methylphenidate (MP), proposed that the orbitofrontal cortex (OFC), part of Stop circuitry, was probably involved in the control of drug rewarding properties, and the loss of OFC inhibitory control over compulsivity and impulsivity could underlie the escalation toward addiction (Volkow et al., 2007). Finally, a structural MRI study in 2011 showed that alcoholics possess a smaller volume of medial frontal cortex and right lateral prefrontal cortex compared to controls, and this reduction was negatively correlated to abstinence duration (Rando et al., 2011).

1.3 The addiction disorders

The definition of addiction disorders refers mainly to two different classes of addiction: substance addiction and behavioural non-substance addiction.

Substance addiction, also called drug addiction, is induce by the neurochemical adaptation and circuitry rewiring of the brain in response to the intake of a molecule(s) known to possess specific pharmacokinetic and pharmacodynamic properties. Typically, the prolonged intake results in a loss of control over molecule(s) use, and whose deprivation induces craving and taking desire also in spite of physical, social and economic negative consequences.

Behavioural addiction, instead, covers a plethora of different disorders sharing similar adaptation and rewiring effects in brain areas of reward system, the same system already demonstrate to be affected by drug addiction, but it differs from the latter for the absence of a chemical(s) acting directly or indirectly

on the brain. Typical non-substance behavioural addiction comprehend pathological gambling, food addiction, internet addiction and mobile phone addiction; among them, only gambling disorders has been included in the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), highlighting the gap still present in the comprehension of behavioural addiction disorders (Zou et al., 2017).

1.4 Food addiction

1.4.1 Excessive food intake and the gut-brain axis

Obesity, defined as a body mass index (BMI) exceeding 30 kg/m^2, is one of the leading cause of death in the world's population, and has reached epidemic proportion with at least 2.8 million people dying each year because of being overweight or obese. World Health Organization (WHO) estimate that, in 2016, 1.9 billion adults were overweight and 600 million were obese worldwide, respectively the 35% and 11% of world's population (World Health Organization, 2017). For the European Union, the healthcare cost for food disorders raise €81 billion, an expenditure that significantly increase considering all obesity-related chronic illnesses (Cuschieri and Mamo, 2016). In fact, excessive adiposity has been reported as a key risk factor for the development of many medical conditions such as cardiovascular disease, hypertension, type 2 diabetes, strokes and cancer (Li et al., 2005), or mental disorders such as major depressive disorders (MDD), bipolar disorders and anxiety (Simon et al., 2006). Despite obesity has been widely described as a multifactorial disease (Grundy, 1998), one of the most important aspect contributing to this pathology is to the capability of high-palatable foods characterized by high amount of fats and/or sugars, typical of more recent western societies, to elicits addictive-like effects, leading to unintended overeating (Davis and Carter, 2009). This mechanism has been ascribed to the absorption signal coming from gut that, through the "gut-brain" axis, alter the DA activity and account for the rewarding properties of palatable food (de Araujo et al., 2012). In particular, the hormones leptin and ghrelin are the most investigated effectors of these signaling. Figlewicz and colleagues have shown that subcutaneous leptin can reverse the conditioned place preference for

sugar in food-restricted rats (Figlewicz et al., 2001) and, more recent evidence demonstrated that leptin induce its hypophagic effect through a mechanism involving DA D2 receptors (D2Rs) in the mesocorticolimbic pathway (Billes et al., 2012). Moreover, intracerebroventricular (i.c.v.) injection of leptin was demonstrated to attenuate the relapse to heroin in food restricted rats (Shalev et al., 2001). On the other hand, ghrelin was demonstrated to induce feeding behaviour when injected in the VTA or in the nucleus accumbens (Naleid et al., 2005) and to increase the extracellular DA in the nucleus accumbens when administered in the VTA (Jerlhag et al., 2007). Moreover, ghrelin also has important effects on two more regions involved in feeding but also in learning and memory, i.e. the hippocampus and amygdala. In fact, it was reported to promote dendritic spine synapse formation and long-term potentiation (LTP) in hippocampus, enhancing spatial learning and memory (Diano et al., 2006). Furthermore, ghrelin suppressed anxiety-like behaviours when injected into amygdala of rats undergoing food abstinence period (Alvarez-Crespo et al., 2012).

1.4.2 Food addiction in animal models

Compulsive overeating as "food addiction" is a relatively recent construct introduced in the last decades to better characterize the pattern of abnormal eating disorders typical of obese and overweight people, firstly proposed by T. Randolph, the founder of environmental medicine, in 1956 (Randolph, 1956). Food addiction has been defined as a chronic and relapsing condition caused by the interaction of complex variables, which induces craving for high palatable food in order to reach energy, pleasure and excitement, or to relieve from negative situations (Imperatori et al., 2016). After initial skepticism on palatable food as an addictive substance, as testified by only six publications between 1950 and 1970, in more recent years this concept gained attention from media and researchers, leading to an increase of scientific literature.

Addiction to highly palatable foods share a plenty of features with drug addiction. Functionally, they are both characterized by the release of neurotransmitters, such as endogenous opioids or DA, in the mesocorticolimbic pathway (Hernandez and Hoebel, 1988; Tanda and Di Chiara, 1998) or by the

activation of the same brain structures (DiLeone, Taylor and Picciotto, 2012). Moreover, they share also symptomatology, such as compulsivity, withdrawal and tolerance (Rogers, 2017). In the last decades, several studies pointed out the similarities between palatable food addiction and drug addiction. For example, rats showed a significant increase of DA and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the nucleus accumbens after 7 days of sucrose intake. Moreover, the pharmacological blockade of DA reuptake or D2Rs induced a significant increase in sucrose intake during test, similar to the effects induced by cocaine (Hajnal and Norgren, 2001; Corrigall and Coen, 1991; Bachtell et al., 2005). Finally, 7 days of restricted access to sucrose solution induced an increase of DAT protein in the nucleus accumbens and ventral tegmental area and of DAT mRNA level in the ventral tegmental area, similarly to cocaine effects (Bello et al., 2003; Zhu and Reith, 2008). Johnson and Kenny in 2010, in order to investigate the effects of restricted or extended access to an obesity-inducing high-calorie diet, i.e. cafeteria diet, compared to no-access, implanted male rats with bipolar stimulating electrode into the posterior lateral hypothalamus and trained them to self-stimulation. After self-stimulation baseline establishment and measured as brain stimulation reward (BSR), rats were exposed to high-calorie foods. During 40 consecutive days of access to cafeteria diet, authors showed that rats with extended access had a marked deficit in reward function, measured as increased threshold for BSR. Similar deficits were previously reported for rats with extended access to cocaine (Ahmed et al., 2002) or heroin (Kenny et al., 2006) intravenous self-administration (S/A), supporting the parallelism between food and drug addiction. After 40 days of exposure, rats were cafeteria-diet deprived and leaved with only normal chows. During this phase of forced abstinence, rats previously exposed to extended access to cafeteria diet showed a persistent elevation in reward threshold, lasting for at least 14 days, a much more longer period compared to reward deficit duration induced by cocaine S/A forced abstinence (~48h) (Markou and Koob, 1991). Furthermore, the compulsivity to cafeteria diet consumption was insensible to cue predicting punishment, i.e. footshock, for rat with a history of extensive access, compared to no access or restricted access. This result

revealed that food seeking resembles drug seeking, showing compulsivity even with negative consequences (Deroche-Gamonet et al., 2004). Finally, molecular analysis found that striatal level of membrane-bound D2Rs was significantly decreased in rats with the extended access to cafeteria diet, a neuroadaptive response to overconsumption of palatable food probably underlying the impairment of reward system function already seen also for substance use disorders (Johnson and Kenny, 2010). Oswald and colleagues obtained similar results in 2011, when they demonstrated that binge-eating prone rats were more resistant to footshock for obtaining highly palatable food than binge-eating resistant controls (Oswald et al., 2011). Binge-eating on food was also reported to induce cross-sensitization, an effect usually observed for drug of abuse (McDaid et al., 2005): in fact, a low dose of amphetamine (0.5 mg/kg ip) increased locomotor activity in rats previously experienced to bingeing on sugar, with no effect in naïve animals (Avena and Hoebel, 2003a). Moreover, even the opposite cross-sensitization was reported, as rats injected with 3 mg/kg ip amphetamine daily for 6 days demonstrated a cross-sensitization for 10% sucrose solution measured as hyperlocomotion and increased sucrose intake, an effect that persists for 5 days (Avena and Hoebel, 2003b). In mice, bingeing on sugar was demonstrated to cross-sensitize to 10 mg/kg ip cocaine and to 20 mg/kg ip morphine, as shown by the increase in locomotor activity after drug injection in mice previously conditioned to sweetened pellets, compared to control groups (Le Merrer and Stephens, 2006). Moreover, rats previously exposed to 12-h access to 10% sucrose solution for 21 days and then leaved in sucrose abstinence for 3 days, showed an increased intake of 9% ethanol solution during test on the following day. These results demonstrated that sugar could be a gateway for bingeing on alcohol, a phenomenon called “gateway effect” (Avena et al., 2004).

In Colantuoni et al. (2002) rats were exposed to sugar bingeing for eight continuous days, and then sugar deprived. After 12 h of sugar fasting followed by naloxone (opiate antagonist, 20 mg/kg ip), rats showed the emergence of anxiety-related symptoms like teeth chattering, forepaw tremors and head shaking. Naloxone after food deprivation reduced the time spent in the open arms during an elevated plus maze test and induced and imbalance in DA/acetylcholine (ACh)

level in the nucleus accumbens. Moreover, authors demonstrated that anxiety-related symptoms emerged spontaneously after 24 h of sugar deprivation, and were observed again at 36 h, resembling opiate withdrawal effects induced by naloxone and suggesting that sugar addiction could be mediated, at least in part, by an endogenous opioids dependence (Colantuoni et al., 2002). Similar effects were reported also in Avena et al. (2008), where extended (28 days) sugar bingeing followed by food abstinence induced anxiety, as indicated by elevated plus-maze test, after 36 h of fasting (Avena et al., 2008). Moreover, the same rats exhibited a significant decrease of extracellular DA and a significant increase of extracellular ACh levels in the nucleus accumbens during abstinence, resembling effects induce by withdrawal from many drugs of abuse, such as morphine, amphetamine, nicotine and ethanol (Rossetti et al., 1992; Bickerdike and Abercrombie, 1997; Rada et al., 1991). An animal model of addiction-like eating behaviour demonstrated that, when stressed, rats prone to binge-eating disorders were more inclined to overeat highly processed food instead of normal chow, similar to stress-induced relapse on drug abuse (Boggiano et al., 2007; Shaham et al., 2000). Finally, a milestone publication in 2007 depicted the possibility that sweet palatable food overcome the addiction to drug, further clarifying previous evidence showing that concurrent access to highly sweetened water (a solution of glucose and saccharin) can alter cocaine self-administration (0.2 mg/kg iv) in rats (Carroll et al., 1989; Carroll and Lac, 1993). In particular, authors reported that a 0.2%-saccharin solution, an intense calorie-free sweetener, as well as 4%-sucrose solution, were preferred to intravenous cocaine self-administration (0.25 mg/kg). The preference for saccharin was stable even with: i) increasing doses of cocaine (up to 1.5 mg/kg), ii) with delayed rewarding effects or iii) with increasing reward price, and it was rapidly and stably acquired even after 3 weeks of extended access (6 h/day) to cocaine self-administration (Lenoir et al., 2007).

As reported above, sugar addiction can go through the different stages of addiction cycle described in the previous paragraph. Besides bingeing on sugar and the subsequent withdrawal accompanied by anxiety, cross-sensitization and gateway effect, animal models could finally experience craving after sugar abstinence, typical of the last preoccupation/anticipation stage. In Avena et al.

(2005), authors trained rats for sugar self-administration in an operant chamber using an FR1 instrumental learning schedule, consisting on the association between pressing on a lever and the delivery of 0.1 mL of 25% sucrose solution for each pressure. Training last 30 minutes daily for all the rats; however, only half of the animals had access to 25% sucrose solution for 11.5 more h each day (extended access). After 28 days of sucrose exposure, animals were sugar fasted for 2 weeks and 24 h later, craving on sucrose was evaluated through a 30-minutes session in the operant chamber equipped with levers. Authors observed that after abstinence rats with extended access exhibit a higher craving compared to non-extended access group, as measured by increased responding on lever during test (Avena et al., 2005). This effect, called incubation of reward craving, is dependent on the duration of withdrawal and has been already observed for drug of abuse such as cocaine, nicotine, heroin, alcohol and methamphetamine (Pickens et al., 2011). Similarly to Avena and colleagues, Grimm and co-authors demonstrated that 15 days (Grimm et al., 2002) or 30 days (Grimm et al., 2005) of abstinence increased craving for sucrose compared to only 1 day, as shown by the increased number of active lever presses during cue-induced reinstatement test the day after withdrawal end. Noteworthy, an early report demonstrating incubation of craving for food in rats and its dependence on withdrawal duration has been published in 1938 by Youtz (Youtz, 1938).

Despite an extensive literature about sugar bingeing and its addiction-like effects on animal models, data regarding binge eating of high fat diet are unclear. As for sugar, limited exposure to fat was able to induce extracellular release of DA in the nucleus accumbens of rats (Liang et al., 2006). Moreover, ad libitum access to fat seem to produces addictive-like behaviours, such as hyperarousal, increased stress and anxiety when ablated from diet (Teegarden and Bale, 2007). However, after limited access (bingeing) to high fat diet for 21-25 days, rats treated with naloxone (3 mg/kg sc) or fat-rich food deprived for 24-36 h did not shown the opiate-like withdrawal features already observed by Colantuoni and colleagues in 2002 or by Avena and colleagues in 2008. These differences between sugar and fat-induced addictive-like properties were explained through the action of the neuropeptide galanin (GAL), a peptide produced in response to

high-fat meal and hypothesized to be involved in the regulation and attenuation of behavioural and neurochemical effects of opiate reward (Zachariou et al., 2003; Hawes et al., 2008). Accordingly, the lack of effects observed in the previously mentioned publications could be caused by galanin activation, which in turn can inhibit opioid-like withdrawal effects usually observed after fasting of sugar bingeing (Avena et al., 2009).

1.4.3 Food addiction in humans

The features of food most likely involved in causing addiction-like effects in humans have been widely investigated by Schultze, Avena and Gearhardt in 2015, based on a parallelism with typical pharmacokinetic features of a drug of abuse. As reported in literature, alteration and processing of a substance are important steps in order to concentrate dose, and therefore increase potency and abuse potential (Henningfield and Keenan, 1993). In line with this previous observation, the level of processing, i.e. addition of fats and/or refined sugars appeared to be a key feature to define a food as liable of addictive-like behaviour. Moreover, another hallmark of addictive agent is the rate of absorption into the blood flow (Verebey and Gold, 1988). In case of a food, the glycemic load (GL), accounting for the blood sugar spike in relation to carbohydrate content, was positively associated to likelihood of developing abnormal eating disorders toward carbohydrates (Schulte et al., 2015).

Food addiction has been defined as a chronic and relapsing condition caused by the interaction of many complex variables that increase craving for specific foods, in order to achieve a state of high pleasure, energy or excitement, or to relieve negative emotional or physical states (Zou et al., 2017). This definition arises from drug addiction criteria listed in the DSM-V (American Psychiatric Association, 2013), that include:

1) Taking the substance in larger amount and for longer period than was intended;
2) Persistent desire or repeated unsuccessful efforts attempt to quit or control the substance use;

3) Large amount of time spent for activity necessary to obtain, to use or to recover from substance use;
4) Important social, occupational, or recreational activities dismissed or reduced because of substance use;
5) Continuative use despite the knowledge of adverse physical or psychological consequences;
6) Tolerance, defines as the need to increase amount of substance to achieve desired effects, or as decrease of effects with continuous use of the same amount of substance;
7) Withdrawal, express as withdrawal syndrome or as the need to use the same or similar substance to relieve or avoid abstinence symptoms.

As for drug users, food addicted usually show a behavioural profile that resembles the aforementioned criteria, supporting analogy between substance use disorders and food addiction. The Yale Food Addiction Scale (YFAS), a 25-items self-report questionnaire designed to identify eating patterns similar to behaviours seen in classic addiction, is based on the above criteria of DSM-V, and has been validated in US and many European Countries both in adults and children (Gearhardt et al., 2009; Gearhardt et al., 2013). Neuroimaging studies revealed that YFAS scores were positively correlated to brain activation in reward areas such as anterior cingulate cortex, orbitofrontal cortex and amygdala in response to anticipation of palatable food intake. Moreover, highly food-addicted patients showed less activation in the orbitofrontal cortex during food intake compared to low addicted. These results were in line with previous evidence of blunted DA response and corresponding weaker reward for substance abusers, associated with a decrease of D2Rs in striatal region (Gearhardt et al., 2011). Furthermore, Wu and colleagues in 2017 reported a decrease of DA transporters (DAT) in striatal region, as well as mRNA downregulation of DAT and tyrosine hydroxylase (TH), the enzyme regulating DA concentrations, in the substantia nigra of post-mortem obese brains vs controls (Wu et al., 2017). Finally, even though vast majority of animal study emphasize sugar-rich foods as the main responsible for binge eating disorders (Avena et al., 2009), a recent paper by Markus and colleagues

highlighted the importance of fat for the palatability related to "addiction-like" problems in humans. In fact, analyzing the relationship between YFAS scores and the kind of food mostly related to own "addictive" features, authors showed that high-fat savory foods (HFSA), such as cheese, chips and meat, and the high-fat sweet foods (HFSW), like chocolate and cake, were indicated as the most problematic, compared to low-fat tasty and only sugar-containing foods. Moreover, both BMI and BDI (Beck Depression Inventory, a validated 21-item self-report questionnaire evaluating depressive symptoms) scores were positively correlated with HFSA and HFSW, bringing back the attention on the possible role of fat on the induction of food addiction and opposing to the negative evidence for fat-rich food withdrawal reported for murine models (Markus et al., 2017). In light of these behavioural and molecular data, both in murine models or humans, is nowadays widely accept to ascribe food addiction to substance use disorders, pathologies characterized by an initial phase of bingeing on substance, and then precipitation to loss of control, tolerance and abstinence, craving and a final relapse to abuse (Koob and Volkow, 2016). Recently, clinical treatment studies showed that more than 85% of individuals relapse to alcohol, nicotine or illicit drug abuse within one year of treatment, and more than two thirds of individuals within weeks or months (Sinha, 2013), clearly demonstrating the lack of long-term successful treatment for addiction disorders.

1.5 Memory processing: from learning to reconsolidation

Memory is a sequence of separate and distinct phases, each one characterized by different molecular and circuital mechanisms responding to specific cognitive requirement. The first phase is the learning phase (I), during which new information are acquired by new experiences and stored as new memory traces. New memories are initially unstable, and need consolidation (II), a neurobiological active phase based on protein synthesis, LTP and receptor trafficking, to be stabilized and strengthened. Moreover, in order to avoid being forgotten, memory need another sustaining process called maintenance (III) that stabilized synaptic connection supporting memory traces. This process also permit memory retrieval (IV) that is the recall of stored information in order to help

guiding individual's behaviour, one of the main purpose of memory (Miller and Springer, 1973). Consolidation cover a time window of several hours, and once stored memory was believed to become static and stable for as long as it persisted (Squire, 1984). For more than 100 years, the idea that once consolidated memories become stably stored in the brain has been a dogma, supported by several line of evidence. In fact, memory was shown to be compromised whether amnestic treatments such us electroconvulsive shock (ECS, Duncan, 1949), protein synthesis inhibitors (PSI, Flexner et al., 1965) or new competing learned information (Gordon and Spear, 1973) were presented after initial learning. On the other hand, memory maintenance could be improved by different treatments, such as strychnine (McGaugh and Krivanek, 1970), cholecystokinin receptors activation (Flood and Morley, 1989) or drug of abuse like amphetamine (Lee and Ma, 1995). Noteworthy, these treatments were effective only during a limited period after memory acquisition but not when delayed in time, leading to the distinction between short-term memory and long-term memory, unstable and sensitive to interferences the former, stable and insensitive the latter (Haubrich and Nader, 2016).

Consolidation theory and the view of retrieval simply as a passive access to previously consolidated memory were challenged since the initial conception. Milestone studies by Misanin, Miller and Lewis and by Schneider and Sherman in 1968 demonstrated that rats had fear memory impairment when they received ECS 24 h after the initial fear conditioning and shortly after memory retrieval through fear-conditioned stimulus (CS) presentation (Lewis et al., 1968; Misanin et al., 1968; Schneider and Sherman, 1968). Moreover, this memory impairment was induced also by competition with new information presented shortly after retrieval (Gordon, 1977), an amnestic treatment effective only when administrated within a defined time window. These amnesic effects were not in line with the previous view of retrieval as a simple passive re-access to static and stable consolidated memory, leading scientists to theorized a new phase of memory processing, i.e. memory reconsolidation (V), that is an active process need to re-stabilize and maintain previously reactivated memory traces (Figure 5).

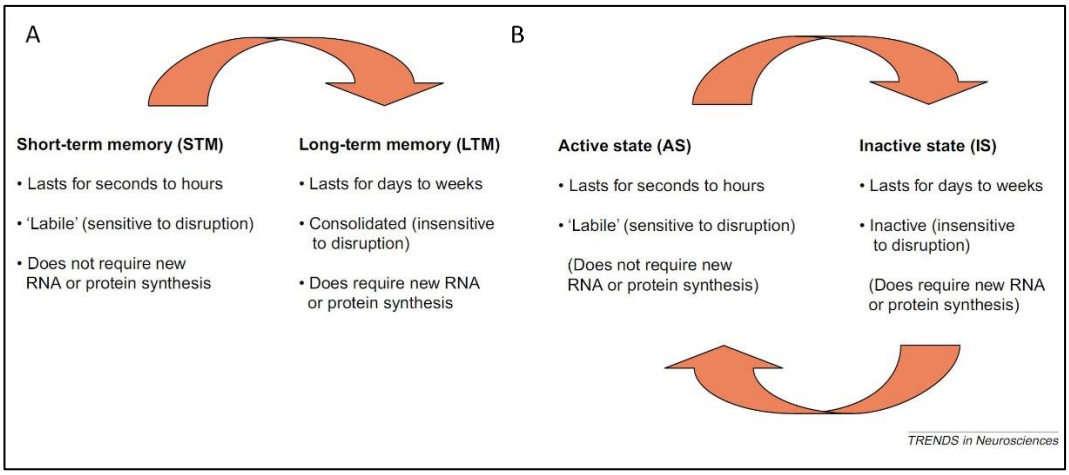

Figure 5: The first and the more recent model of memory processing. (A) The first model, with the initial learning codified in a short-term memory that will consolidate in a long-term stabile and unchangeable memory trace. (B) The new model of memory processing, where after the initial codification in a short-term memory (active state) the memory is then stabilized in a long-lasting inactive state (long-term memory). However, a retrieval session can return memory trace from the inactive state to a new active state, requiring a new reconsolidating phase to return a stable memory engram. Taken and adapted from Nader, 2003.

1.6 Memory reconsolidation

After its initial conceptualization, memory reconsolidation received modest investigation for many years, until a milestone publication in 2000 by Nader and colleagues (Nader et al., 2000). In this paper, authors used a well-characterized behavioural protocol, i.e. auditory fear conditioning, and anisomycin (ANI), an antibiotic known for its amnesic effect on long-term auditory fear memory through inhibition of protein synthesis. After fear memory retrieval with CS, rats received bilateral infusion of ANI into the basolateral amygdala, a brain region important for emotional information processing. The treatment with ANI was able to impair long-term, but not short-term, memory retention as shown by test 24 h after retrieval. However, the treatment was effective only within a defined time window and specifically after retrieval, as the same treatment 6 h after CS presentation or without CS presentation did not inhibit fear memory (Nader et al.,

2000). Hence, the same evidence supporting consolidation as a stabilization process after learning supported also reconsolidation as a necessary restabilization phase after retrieval (Haubrich and Nader, 2016). In the past 10 years, the study of reconsolidation have been extended to numerous experimental paradigms and different animal species, as summarized in Table I. The protocol for memory reconsolidation study typically go through the different phases described above: after an initial training phase, memory are reactivated, manipulated and finally tested for retention. Reactivation is usually induced by exposure to CS (unreinforced retrieval) and, more rarely, also to US (reinforced retrieval). In general, memory reconsolidation has been demonstrated rarely through its enhancing (Stern and Alberini, 2013), and much more often, through its inhibition (Lee, 2009; Tronson and Taylor, 2013). Whether behavioural (Xue et al., 2012), pharmacological (Nader et al., 2000) or genetic (Lee et al., 2004) manipulations applied after retrieval cause changes in behaviour, we can conclude that the active process of reconsolidation has been addressed.

1.6.1 Molecular mechanisms in memory reconsolidation

Different molecular mechanisms participate, at different level, to memory reconsolidation process. As already mentioned, protein synthesis is a key process for both consolidation and reconsolidation (Tronson and Taylor, 2007). Moreover, N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and β-adrenergic receptors (β-ARs) have been reported as key targets for pharmacological reconsolidation inhibition (Milton et al., 2008a; Bhattacharya et al., 2017; Fricks-Gleason and Marshall, 2008). In addition, transcription factors (TFs) play a crucial role in memory reconsolidation. Cyclic AMP-response element binding protein (CREB) disruption in prefrontal cortex, hippocampus and amygdala is reported to impair memory reconsolidation of both auditory and contextual fear memory (Kida et al., 2002). Lee and colleagues in 2004 described a double dissociation between brain-derived neurotrophic factor (BDNF) and the TF zinc finger protein 268 (Zif268) involvement in consolidation and reconsolidation. Rats injected directly in the dorsal hippocampus with BDNF antisense oligodeoxynucleotides (ASO) 90

minutes before contextual fear conditioning resulted in a short-term (24 h after) and long-term (7 days after) impairment of contextual fear memory. This effect was not observed when infusion was done 90 minutes before memory retrieval or with Zif268 ASO, indicating a specific requirement of hippocampal BDNF for fear memory consolidation. Conversely, infusion of Zif268 ASO 90 minutes before memory reactivation resulted in amnesia during a post-retrieval LTM test, an effect that was not observed in the absence of a retrieval session or with infusion of BDNF ASO, indicating the specificity of Zif268 for fear memory reconsolidation (Lee et al., 2004). However, Zif268 is crucial not only for hippocampal-dependent memory reconsolidation, but also in basolateral amygdala for auditory fear memory (Hall et al., 2001) and for drug-associated memory reconsolidation (Milton et al., 2008b; Théberge et al., 2010). Another dissociation in consolidation and reconsolidation involvement has been demonstrated for C/EBPβ. While this TF is required in hippocampal-dependent consolidation, but not reconsolidation, of inhibitory avoidance (IA) learning, in amygdala it is involved in the reconsolidation, but not consolidation, of IA memory (Tronel et al., 2005). Lastly, important roles in memory reconsolidation are played also by kinases, such as extracellular signal-regulated kinase (ERK), protein kinase A (PKA), protein kinase C (PKC) or mechanistic target of rapamycin (mTOR). In particular, the mTORC1 (mTOR complex 1) cascade and its suppression by rapamycin has been linked to memory reconsolidation inhibition in different experimental paradigms, like contextual fear memory (Blundell et al., 2008), auditory fear memory (Tedesco et al., 2014b), alcohol-associated (Barak et al., 2013) and cocaine-associated (Wang et al., 2010) instrumental memory. These evidences highlight the importance of this cascade and its downstream effectors p70S6K and rpS6 in memory reconsolidation, related to its role in *de-novo* protein synthesis locally in active synapses (Neasta et al, 2014; Pirbhoy et al., 2017) (Figure 6).

All the information available nowadays about memory reconsolidation, from animal species to experimental tasks and molecular mechanisms, shed light on the fundamental role of this process. Act on the stability of memory, by an evolutionary point of view, it serves as an adaptive update mechanism allowing

for the integration of new information on the original memory traces (Hupbach et al., 2007) or even to strengthen them (Inda et al., 2011). However, such a delicate function should have boundary conditions defining the features of a retrieval session, in order to control tightly the parameters that induce labilization and thus destabilization of mnemonic engrams.

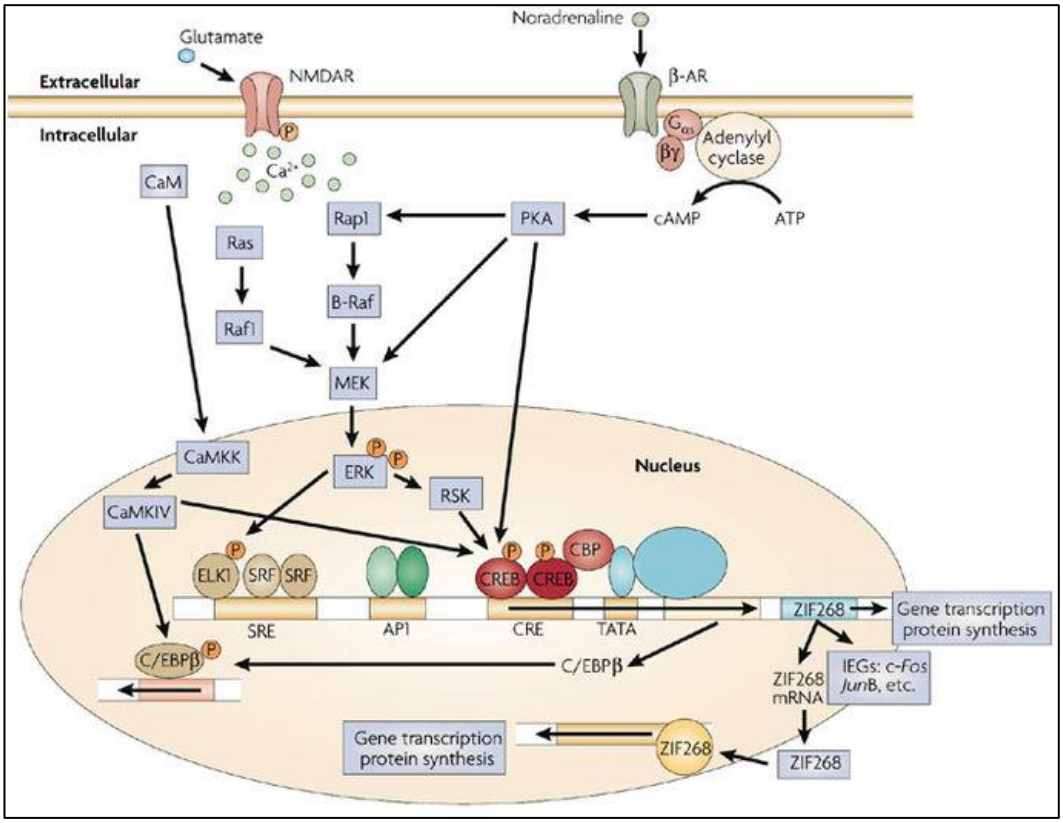

Figure 6: Important pathways and molecular markers for memory reconsolidation. β-ARs and NMDARs are key receptors in molecular mechanisms of memory reconsolidation: through the influx of calcium ions, they could activate the MAPK/ERK pathway or protein kinase A (PKA), ending with the activation of cAMP response element-binding site (CREB) that stimulates the gene transcription of important protein, such as cFos, JunB or Zif268. Taken and adapted from Tronson and Taylor, 2007.

Table I: Experimental paradigms, amnestic treatments and species of reconsolidation studies. Taken and adapted from Haubrich and Nader, 2016.

Experimental paradigm	
Auditory fear conditioning	Nader et al., 2000
Contextual fear conditioning	Debiec et al., 2002
Episodic memory	Hupbach et al., 2007
Habituation	Rose and Rankin, 2006
Inhibitory avoidance	Anokhin et al., 2002; Litvin and Anokhin, 2000
Instrumental learning	Hernandez and Kelley, 2004; Tedesco et al., 2014a
Incentive learning	Wang et al., 2005
Positive reinforcement	Flavell et al., 2011
Memory for drug reward	Lee et al., 2005; Milton et al., 2008a; 2008b
Object recognition	Kelly et al., 2003
Spatial memory	Suzuki et al., 2004
Amnestic treatment	
Antisense oligonucleotides	Lee et al., 2004; Taubenfeld et al., 2001
Inhibition of kinase activity	Duvarci et al., 2005, Kelly et al., 2003
Inducible knockout mice	Kida et al., 2002
Interference by new learning	Hupbach et al., 2007, Walker et al., 2003
Potentiated reconsolidation by increase in kinase activity	Tronson et al., 2006
Protein-synthesis inhibition	Nader et al., 2000
Protein knockout mice	Bozon et al., 2003
Receptor antagonists	Kindt et al., 2009; Suzuki et al., 2004
RNA synthesis inhibition	Sangha et al., 2003
Species	
Chick	Anokhin et al., 2002
Crabs	Pedreira et al., 2002
Honeybees	Stollhoff et al., 2005
Humans	Hupbach et al., 2007; Walker et al., 2003
Mice	Kida et al., 2002
Rats	Nader et al., 2000
Sea slugs	Child et al., 2003; Lee et al., 2012
Sheep	Perrin et al., 2007

1.6.2 The boundary conditions of memory reconsolidation

Not all the retrieval sessions can induce reactivation of the memory; there are important parameters, related to the intrinsic characteristics of the consolidated memory traces that determine whether that reactivation, destabilization and

eventually reconsolidation will take place. These parameters, called “boundary conditions”, have received increasing attention in the last years as important determinants of whether memory is more or less susceptible to be reactivated and, possibly, disrupted (Auber et al., 2013).

Two of the most important boundary conditions are memory age and retrieval duration. As reported in Frankland et al. (2006), when ANI was injected into the dorsal hippocampus of mice after memory retrieval by short context re-exposure, the expression of a recent (1-day old) contextual fear memory was blocked during a test 24 h later. The same infusion was not effective with a 36-day old memory or when context re-exposure (retrieval) was omitted. Conversely, infusion of ANI in the anterior cingulate cortex after short or extended context re-exposure was ineffective in blocking the expression of both 1-day and 36-day old contextual fear memory. Nevertheless, when ANI was injected systemically after an extended retrieval session, memory was disrupted in the test 24 h later. These results indicated that, while recent memory are located in more limited subcortical regions, like hippocampus, old memory are more widespread in the cortex and PSI need to be systemically injected to be effective. Moreover, in addition to the age of a memory, even the strength of the retrieval session is a boundary condition that can determine whether memory will be reactivated (Frankland et al., 2006). Bustos and colleagues obtained similar evidence in 2009, when they analyzed the effects of systemic midazolam (MDZ), a benzodiazepine effective in memory reconsolidation disruption, on contextual fear memory reconsolidation. They reported that in order to prevent the reconsolidation of increasingly old memories, they need to increase, in parallel, the duration of retrieval session and/or the MDZ dose after retrieval. However, an excessively prolonged non-reinforced retrieval session can induce extinction, defined as the establishment of a new competing memory trace where the originally association between CS and US is now replaced by CS-no reinforcement association (Bouton, 2004). Therefore, MDZ treatment following extinction resulted in the disruption of extinction consolidation, rather than reconsolidation inhibition (Bustos et al., 2009).

In the elegant study by Wang et al. (2009), authors reported that also the length of training phase, and so the memory strength, is a determining parameter for

memory reactivation. In fact, 10 tone-shock pairings created a memory trace stronger to be reactivated and inhibited by ANI injection into basolateral amygdala then a single pairing. However, the inability of ANI to induce behavioural impairment was prevented only when retrieval was performed at 2 and 7 days after strong training. When memory was reactivated 30 or 60 days after training, ANI was able to induce reconsolidation deficits. Moreover, in line with Frankland et al. (2006), when dorsal hippocampus was electrolytic lesioned before strong training, ANI infusion into basolateral amygdala following retrieval was able to block post retrieval-long term memory even at 2 days after training, indicating the fundamental and time-limited role of dorsal hippocampus in inhibiting re-access to strong memory traces. Finally, the authors identified GluN2B-containing NMDARs (GluN2B) as main regulators of the strength need to reactivate memory, with a decreased receptors level 2 days after strong training preventing reactivation, and a return to basal level after 60 days or following dorsal hippocampal lesion, allowing reactivation (Wang et al., 2009). Other boundary conditions reported to influence memory retrieval and reconsolidation are the reactivation context (Hupbach et al., 2008), the specificity of CS for the re-accessed memory (Debiec et al., 2006) and the moment during which it is reactivated, i.e. during waking or during sleep (Diekelmann et al., 2011). Finally, it is noteworthy that the majority of boundary conditions are related to the feature of novelty: in fact, predictability seem to be one of the most important constraint for memory reconsolidation (Pedreira et al., 2004). Furthermore, the prediction error signal, defined as the signal elicited by the mismatch between the expected event and what actually happen, has been hypothesized as a key factor of a reminder for its capability in re-accessing and thus labilizing previously consolidated memory (Fernández et al., 2016).

In conclusion, understanding the boundary conditions for memory reconsolidation is critical because targeting reconsolidation process has been proposed to be a potential treatment for maladaptive memory-related diseases, such as PTSD or substance use disorders. Therefore, if molecular or cellular markers linked to boundary conditions will be clearly identified, they could be helpful to recognize

the optimal retrieval condition and predicting success or failure of memory reactivation procedure.

1.6.3 Pavlovian vs instrumental memory

Pavlovian (or classical) and instrumental (or operant) conditioning are two important concepts of behavioural neuropsychology, concerning different methods of learning and behavioural modulation in animal models.

Pavlovian conditioning was firstly theorized by the Russian physiologist Ivan Pavlov in 1902, observing the salivation reflex of his dog in response to food (Pavlov, 1927). In fact, the classical conditioning consists in the presentation of a neutral signal, like a sound, an odor or a light before an unconditioned stimulus (US). An US is a stimulus that possess positive (like food) or negative (like a mild shock) reinforcing properties *per sé* able to generate a reflex response. After a period of neutral signal followed by US presentation, the neutral signal will be associated to the US and will generate the reflex response on its own, without the presentation of the original US. Therefore, with the classical conditioning it is possible to couple a neutral stimulus with a reinforcement, generating a conditioned response (CR, salivation for food or freezing for footshock) toward the neutral signal that will eventually became a conditioned stimulus (CS) (McSweeney and Murphy, 2014).

Instrumental conditioning instead has been firstly described by the American psychologist Burrhus Frederic Skinner in 1938 (Skinner, 1938). Here, the association is between an action and a succeeding positive or negative outcome, and the experiments are usually performed in a conditioning box, also known as Skinner box (Figure 7). A positive outcome, namely a reinforcement, will increase the motivation toward repeating the action, while a negative outcome, namely a punishment, will weaken the behaviour, decreasing the probability of the action being repeated. Reinforcement can be either positive or negative: a positive reinforcement, like food, is something that an individual finds rewarding, while a negative reinforcement consists in the removal of an uncomfortable situation, like being exposed to the sunlight for a rat. Avoiding the uncomfortable situation generates a sort of “reward” for the individual, strengthening that behaviour.

For rat or mouse conditioning, it is possible to use different patterns of reinforcement in order to modulate the speed and strength with which an instrumental conditioning is acquired. These patterns are experimentally called schedules, and can be fixed- or variable-ratio schedules. With a fixed-ratio (FR) schedule, the reinforcement is acquired following a fixed number of time the action is completed, like 10 lever presses to obtain a pellet (FR10); conversely, with a variable-ratio (VR) schedule, the reinforcement is acquired after a variable number of time the action is completed, like between 12 and 25 lever presses to obtain a pellet (VR20). Moreover, there are also fixed- or variable-interval schedules, during which the reward is obtained after a fixed or variable interval of time. These schedules have different effects on the rate of responding and the rate of extinction of the trained animals, with variable-ratio schedules supposed to create more resistant memory traces because of unpredictability of reinforcement (McLeod, 2007). However, also the number and duration of the training trials carried out during the learning phase regulate the memory strength, rendering the memory trace more or less susceptible to changes mediated by behavioural or pharmacological manipulations.

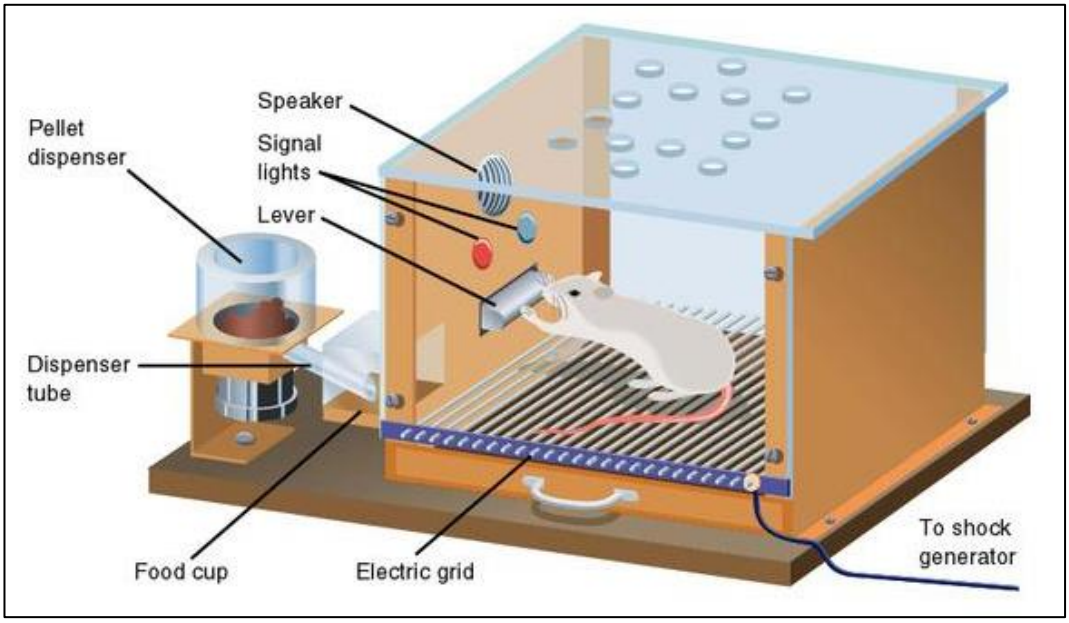

Figure 7: A typical conditioning box. Coupling the lever pressing (on the center of frontal wall) with pellets delivery (on the right of the frontal wall) in presence or in absence of a CS can induce respectively pavlovian instrumental or pure

instrumental conditioning. Here it is also possible to produce a classical conditioning, coupling a tone or a light stimulus (CS, above the lever) with a pellet delivery or a footshock (delivered by the electric grid floor). Taken from McLeod, 2007.

1.6.4 Sucrose instrumental memory reconsolidation

Memory reconsolidation for high palatable food has been investigated using the experimental protocols typically applied to study drug addiction memory. One of these protocols has been already mentioned above in the paragraph 1.4.2 and used in Grimm et al. (2002, 2005) and Avena et al. (2005). It follows the normal progression of drug addiction, i.e. an initial bingeing on sucrose, a subsequent withdrawal phase with incubation of reward craving and finally sucrose craving and taking evaluation through memory extinction and reinstatement tests. In Grimm et al. (2002) after 10 days of training to lever presses for sucrose associated to tone + light cues, rats underwent 1 or 15 days of withdrawal followed by extinction sessions and memory reconsolidation test. Authors observed that, resembling cocaine memory, sucrose instrumental responding associated to CS exposure was more resistant to erasure after 15 days than 1 day of withdrawal, demonstrating the incubation of craving for sucrose (Grimm et al., 2002). Then, Hernandez and colleagues have done a similar study in the same year on sugar instrumental memory consolidation and reconsolidation. To study the requirement of protein synthesis for food memory consolidation, researchers trained rats to self-administer sugar pellets with a daily session of 15 minutes for 12 days. During the session, rats in the operant chambers received a sugar pellets every two lever presses on the active lever (FR2). A second, inactive lever had no consequence. A red light (CS) was associated to sugar pellets delivery. When ANI (62.5 μg) was injected directly in the nucleus accumbens core of rats immediately after each one of the first five daily trials, authors reported a significant impairment of learning, concluding that protein synthesis is required for sugar memory consolidation. Same injection in the nucleus accumbens shell did not induce significant effect. Instead, when ANI was injected in nucleus accumbens

core immediately after the last trial sessions (day 9, 10 and 11), researchers did not observed learning impairment, concluding that protein synthesis was not need for food memory reconsolidation (Hernandez et al., 2002). However, the experimental protocol did not contemplate a distinct withdrawal or retrieval sessions, with the latter necessary for the labilization of the memory trace and hence for memory reconsolidation manipulation. Similar results were obtained by the same authors in a following report. Here, researchers treated rats with 20 mg/kg ip ANI during the last sessions of sugar instrumental training and observed a decrease in responding on levers that was lately addressed to the development of conditioned taste aversion for sugar rather than to sugar memory reconsolidation inhibition. In this case, memory retrieval after training was induced by non-contingent exposure to sugar boluses consumption (US without CS); however, the protocol still lack of a clear withdrawal period that could induce craving incubation. Therefore, the lack of reconsolidation inhibition was referred to a possible overtraining that strengthened memory trace to a protein synthesis-independent level - a condition that can explain also the lack of results in Hernandez et al., 2002 - or to the inability of the current approach using US as retrieval to disrupt memory reconsolidation (Hernandez and Kelly, 2004).

Two more publications in 2005 investigated the incubation of craving for sucrose: Grimm et al. extended the previous evidence reporting that craving for sucrose was increased after 30 days of fasting, compared to 1 or 7 days (Grimm et al., 2005). On the other hand, Avena et al. reported that rats with an history of extensive sucrose bingeing showed enhanced intake after 14 days of abstinence, compared to rats having short access to sucrose, suggesting that sucrose bingeing could induces behavioural changes indicative of addiction-like alterations (Avena et al., 2005). Reconsolidation of instrumental learning for sucrose was firstly demonstrated by Wang and colleagues in 2005. Using a protocol of incentive learning, authors initially associated a sucrose solution to left lever and sucrose pellets to right lever (or vice versa) inside an operant chamber (training phase). Twenty-four hours later, rats were *ad libitum* exposed to sucrose for reward devaluation followed by intra-basolateral amygdala infusion of vehicle (VEH) or ANI and, the day after, tested for memory consolidation. After consolidation test,

groups were re-exposed to the devaluated reward, treated with the opposite drug (before VEH, then ANI (V/A) or before ANI, then VEH (A/V)) and the day after, tested for reconsolidation. As expected, ANI inhibited consolidation and thus reward devaluation during the first test, compared to VEH. The second test instead demonstrated that even memory reconsolidation is ANI-sensitive and thus dependent on protein synthesis, as reward re-exposure (retrieval session) followed by ANI blocked incentive learning and induced reconsolidation inhibition at the final test (Wang et al., 2005).

Instrumental memory reconsolidation for sucrose has been shown to depend on β-ARs through their inhibition with propranolol injection (10 mg/kg ip) immediately after retrieval (Diergaarde et al., 2006; Milton et al., 2008b); moreover, it has been disrupted also with MK-801 injection (0.1 mg/kg ip) 30 minutes prior to retrieval (Lee and Everitt, 2008b). In the latter study, memory reactivation was performed by exposure to an experimental session identical to training trials, except for the lack of sucrose US. The importance of retrieval setting was investigated by the same authors in another publication of 2008: here, Lee and Everitt used an experimental protocol similar to the previous study, but reactivation was performed with CS presentation both contingently and non-contingently to active lever pressing, besides US absence. Interestingly, in addition to confirm pre-reactivation MK-801 efficacy in successive reconsolidation inhibition, they reported that only when CS presentation was given contingently upon active lever press memory was actually reactivated, and MK-801 could inhibit reconsolidation during test. Conversely, when CS was presented in the absence of levers, MK-801 was ineffective to block reconsolidation, whether injected before or after reactivation. These results shed light on the importance of salient memory-evoking stimuli presentation for the targeting of memory reconsolidation as therapeutic tool for addictive behaviours (Lee and Everitt, 2008a). A third paper by Lee and Everitt in 2008 demonstrated that Pavlovian representation at the bases of a Pavlovian-instrumental transfer (PIT) undergo NMDARs-dependent reconsolidation, as MK-801, but not propranolol, injection 30 min before CS re-exposure reduced instrumental responding in a subsequent test (Lee and Everitt, 2008c).

Mierzejewski and colleagues in 2008 were able to alter appetitive operant conditioning for saccharin through subcutaneous injection of the protein synthesis inhibitor cycloheximide (CHX). After self-administration training, during which rats associated active lever presses with a CS and saccharin rewards, animals underwent multiple extinction sessions with subcutaneous CHX 3mg/kg or VEH treatments immediately after each session. Extinction could be 5-min or 30-min long and was characterized only by operant box exposure (context) equipped with levers, without CS or US presentation. Authors observed that with 5-min extinction sessions animals extinguished operant behaviour faster than controls. Conversely, with 30-min sessions animals did not extinguished, rather they increased operant responding on active lever. These results highlighted the importance of retrieval session features: considering the first extinction session as a brief (5 min) or extended (30 min) reactivation, researchers concluded that a 5-min exposure to context + levers followed by CHX was able to inhibit instrumental memory reconsolidation, while a 30-min exposure followed by CHX inhibited the consolidation of the new extinction memory. Noteworthy, extinction sessions were characterized by novelty, as differently from training it did not exposed rats to CS or US (Mierzejewski et al., 2008). Nevertheless, the same authors in a 2009 reported that, following the same experimental protocol, instrumental memory did not underwent reconsolidation inhibition through CHX injection (Mierzejewski et al., 2009). However, here extinction sessions were replaced by 5-min long self-administration trials including US and CS presentation, thus losing the features of novelty previously reported. Instead, this last protocol could be considered simply as a long-lasting self-administration phase, which was already reported as *de novo* protein synthesis-independent by Hernandez and Kelly in 2004.

The balance between reconsolidation and extinction in an appetitive instrumental memory task was assessed by Flavell and Lee in 2013. After a 5-day training during which rats learned to associate active lever presses to sucrose and CS, and 3 days of abstinence, animals received MK-801 (0.1 mg/kg ip) or vehicle 30 min before memory retrieval. Different groups underwent different retrieval sessions: pure instrumental group was reactivated only with lever pressing for a maximum

of 10 or 50 pressures, while Pavlovian + instrumental group was reactivated with CS presentation upon active lever responding. This last was further divided in three subgroups on the bases of CS exposure during reactivation: the first subgroup was allowed to press lever to receive up to 10 CS, the second up to 30, and the third up to 50. A separate group of rats was treated with MK-801 or vehicle in the home cage as a control (no-retrieval group). Memory test was performed 48 h later. Results demonstrated that MK-801 affected behaviour at test only when injected before 10 or 50 CS retrieval session, without difference compared to vehicle when injected before no-retrieval, before 30 CS presentation and 10-presses or 50-presses pure instrumental retrieval groups. Specifically, when injected before 10 CS presentation, instrumental memory was reported to be impaired during test, while it was enhanced when MK-801 was injected before 50 CS exposure. Based on previous literature about fear and other appetitive memories, authors concluded that a brief CS exposure triggered destabilization leading to reconsolidation, and the NMDARs antagonism inhibited this process leading to reconsolidation inhibition. Conversely, prolonged exposure to CS induced consolidation of a new extinction learning, and its inhibition with MK-801 treatment leaded to the reconsolidation and strengthening of the original memory trace (Flavell and Lee, 2013).

In the studies reported so far, only Mierzejewski et al. (2008) showed that pure instrumental memory reconsolidation could be inhibited by PSIs, while Flavell and Lee (2013) reported that memory reconsolidation is not affected by MK-801. However, Exton-McGuinness and colleagues in 2014 firstly demonstrated that also MK-801 could inhibit pure instrumental memory reconsolidation. In this paper, authors demonstrated that a well-learned 10-day pure instrumental memory was correctly reactivated with a VR20 retrieval session. During training, pellets were administrated under an FR1 schedule; however, during reinforced retrieval session, the number of active lever presses need to acquire a reward was variable and, specifically, rats received sucrose pellets after a mean of 20 active lever presses, in a range between a minimum of 12 and a maximum of 28 presses. Thirty minutes before reactivation, rats were treated intraperitoneally with MK-801 0.1 mg/kg or vehicle. MK-801 prior to VR20-reactivation significantly

decrease instrumental responding during a 30-min extinction test the day after retrieval. As control, separate groups of rats received a brief non-reinforced retrieval session, a FR20-reinforced retrieval session and a no-reactivation session. In all these control groups, MK-801 before memory reactivation did not impaired reconsolidation during test, suggesting that memory trace was not correctly destabilize. Moreover, authors proved that this effect was specific for reconsolidation inhibition and not referable to a devaluation of reward or to a reduction in general activity or motivation (Exton-McGuinness et al., 2014). Furthermore, Exton-McGuinness and Lee in 2015 expand the evidence of pure instrumental memory reconsolidation to 2-d weakly trained lever-pressing memory. Here, authors showed that MK-801 (0.1 mg/kg ip) 30 min before a VR5 reactivation session was able to impair reconsolidation in a 30-min test the day after. As before, they demonstrated that a non-reinforced or a fixed-ratio (FR1) reactivation did not exert the same effect as a variable-ratio session. Finally, they demonstrated also the involvement of accumbal NMDARs and D1Rs in the memory reconsolidation process. Immediately before VR20-reactivation, AP-5 (NMDARs antagonist) and SCH23390 (D1Rs antagonist) were injected into the nucleus accumbens individually or combined. As observed in the test 24 h later, only the co-infusion was effective in disrupting reconsolidation, while intra-accumbens AP-5, SCH23390 or MK-801 alone did not reduce instrumental responding. In conclusion, authors pointed out the need of a shift in reinforcement contingency during retrieval, which can lead to a prediction error signal assumed to be a critical determinant for the labilization of a consolidated memory trace (Exton-McGuinness and Lee, 2015).

To conclude, it is important to note that some recent studies have analyzed also the possibility to disrupt memories applying an extinction training after reactivation, using the memory-updating function of the reconsolidation process to investigate the possible therapeutic aspect of erasing a maladaptive conditioned response. Flavell, Barber and Lee in 2011 demonstrated that extinction applied 1 hour after a reactivation session induced a long-lasting impairment in the acquisition of a new instrumental response, using a protocol of reactivation + extinction demonstrated to be effective in attenuating fear conditioning (Monfils

et al., 2009). Initially, rats were trained for 20 minutes over 9 days to nosepoke into a food magazine to receive sugar pellets with an FR1 schedule, and the nosepokes were coupled with the presentation of a light stimulus (CS). Twenty-four hours after training, memory was reactivated by a 10-min extinction training, identical to training trials except that nosepokes did not delivered pellets. One or six hours after retrieval, memory was extinguished with a 60-min extinction training, and tested for the acquisition of a new instrumental response on levers at 1, 2, 5, 8, 13, 20 and 27 days later. During tests, pressing the active lever on the opposite side of the training CS resulted in the CS light stimulus, while a nosepoke or pressing the inactive lever in the same side of CS resulted in no consequence. In particular, at test day 1, 2, 3 and 5 CSs were presented with an FR1 schedule, while at day 13 and 20 the schedule was FR3 to increase the probability of spontaneous recovery. The final test at day 27 was a reinstatement test, as 10 sugar pellets were available in the training context before test. When presented 1 h after retrieval the extinction session was able to impair the acquisition of a new instrumental response during tests. Moreover, memory was impaired until the last reinstatement test, demonstrating that it did not recover during subsequent tests nor reinstated by sucrose exposure. However, as for fear memory (Nader et al., 2000), reconsolidation has a limited time windows, as applying extinction 6 h after reactivation did not result in memory impairment. Finally, researchers noted some differences compared to fear memory; in fact, the simple exposure to the training context was able to reactivate correctly the memory, as an extinction session after context exposure resulted in a memory impairment similar as after CS retrieval. This was in contrast with previous findings (Monfils et al., 2009), arguing that in appetitive setting the conditioning context could act as an “occasion setter”, contributing to the ability of the CS in modulating the behaviour. Moreover, an injection of d-cycloserine (DCS), a partial NMDARs agonist, instead of retrieval, 1 h before extinction did not impair the new response acquisition during tests, in contrast with previous evidence for fear memory (Ledgerwood et al., 2004). DCS was supposed to enhance extinction, and the lack of effects leads the authors to posit that the effect observed after reactivation followed by extinction was due to the specific effect of

retrieval in inducing destabilization, rather than potentiating extinction learning (Flavell et al., 2011). Notably, DCS was able to enhance extinction of cocaine self-administration (Thanos et al., 2011). Finally, a cued retrieval session followed by extinction within six hours was able to impair heroin seeking in both rats and humans (Xue et al., 2012), demonstrating that this procedure could have significant therapeutic impacts for the treatment of maladaptive memory diseases like PTSD and substance or non-substance addiction.

1.7 The concept of metaplasticity

In the late 1990s, studying the mechanisms of long-term potentiation (LTP) and long-term depression (LTD), basic for the induction of long-lasting synaptic plasticity strengthening (the former) or weakening (the latter), W.C. Abraham and M.F. Bear defined a new concept, that they called metaplasticity. They found that weak synaptic stimulations, even causing only a transient or null potentiation *per sé*, were able to strongly influence future synaptic events, like potentiation or inhibition of LTP and LTD triggered by a subsequent stimulation. This effect was summarized as "*the plasticity of synaptic plasticity*" or, as mentioned above, as metaplasticity (Abraham and Bear, 1996). Since the beginning, metaplasticity was demonstrated to be mediated primarily by ionotropic and metabotropic glutamate receptors and their downstream cascades (Abraham and Tate, 1997). For example, aberrant metaplasticity seems to be involved in neurodegenerative disorders such as Alzheimer's disease (AD). In AD, amyloid-β oligomers, one the main causes of the disease, reduces glutamate reuptake causing an increased activation of NMDARs, especially through GluN2B, possibly leading to excitotoxicity (Li et al., 2011). Moreover, GluN2B receptors have been linked also to Parkinson's (Nash et al., 2004) and Huntington's disease (Li et al., 2004).

Besides synaptic and cellular metaplasticity, more recently metaplastic mechanisms have been hypothesized to underlie also behavioural conditions, conceptualized as "behavioural metaplasticity" and comprising long-lasting behavioural alteration resulting from metaplastic triggering events such as stress (Schmidt et al, 2013), enriched environment exposure or sensory deprivation (Abraham, 2008). Moreover, the increased synaptic plasticity mediated by drug

addiction has been suggested to be mediated possibly by metaplasticity-dependent LTP (Hulme et al., 2013). In fact, amphetamine and ethanol ease the induction of LTP through NMDARs in the dopaminergic neurons of the ventral tegmental area (Ahn et al., 2010; Bernier et al., 2011), while cocaine and methamphetamine induce long-term modulation of LTP magnitude in the CA1 area of hippocampus (Thompson et al., 2004; Swant et al., 2010). Moreover, cocaine exposure generates silent synapses in the nucleus accumbens, which then potentiate during abstinence (Hulme et al., 2013). Cocaine also enhanced excitatory strength in dopamine neurons of ventral tegmental area at 1, 7, 21 and even 90 days after the last self-administration session; interestingly, also sucrose self-administration was able to induce the same excitatory enhancement, though limited to 7 days after the last session (Chen et al., 2008). This last evidence suggests that even food addiction could depend on metaplastic neuromodulation.

Acting through glutamatergic receptors for LTP and LTD modulation, it was hypothesized that metaplasticity could in turn influence also memory processing and the stages of consolidation and reconsolidation. A comprehensive and detailed review by Finnie and Nader in 2012 speculate on a number of cellular and molecular metaplastic mechanisms regulating memory destabilization and reconsolidation. One of the most emphasized metaplasticity-related process was the downregulation of NMDARs containing GluN2B or the increased GluN2A/GluN2B ratio after strong training, as the greater calcium transmission of GluN2B compared to GluN2A allows for synaptic destabilization mechanisms that must be blocked for long-term maintenance of relevant memory traces. Conversely, GluN2B is necessary for memory destabilization process and its trafficking from and to the post-synaptic membrane underlie activity-dependent remodeling of synaptic stability. Moreover, GluN2B can promote LTP by itself through a mechanism involving C-terminal cytoplasmic tail (Foster et al., 2010) or through the induction of CaMKII autophosphorylation, a mechanism required in many forms of long-lasting synaptic changes (Zhou et al., 2007).

Another important molecular marker for plasticity, metaplasticity and memory processes are the calcium-permeable GluA1-containing AMPARs. Their membrane insertion after synaptic stimulation can lasts days after behavioural

experience; moreover, trafficking, stability and activity are regulated by PKA-mediated phosphorylation, and PKA along with GluN2B and low voltage-gated calcium channels (LVGCCs) interact with GluA1 for destabilization regulation (Finnie and Nader, 2012).

Finally, also metabotropic glutamate receptors seem to be involved in long-lasting plasticity mechanisms. Bortolotto et al. (2005) demonstrated that, after LTP, mGluR5 is fundamental for a molecular switch that render a subsequent LTP induction insensitive to mGluR antagonist MCPG (Bortolotto et al., 2005). Abraham in 2008, suggested that group I mGluRs (mGluR1 and mGluR5) could ease LTP induction by AMPARs and NMDARs trafficking and activity facilitation; moreover, mGluRs seem to be involved also in local synaptic protein synthesis through the stimulation of mTORC1 signaling cascade and its downstream effectors such as eF2, eF1α, CaMKII and phosphorylated-rpS6 (rpS6P) (Abraham, 2008). More recently, Marton and colleagues in 2015 proposed a metaplastic role for mGluR5 and its binding protein Homer1A, a plasticity-related immediate early gene, in long-lasting potentiation of striatal synapses, involving prior stimulation of D1Rs and acting through enhancement of NMDARs current, an effect hypothesized to underlie behavioural effect of cocaine self-administration. Moreover, interaction between mGluR5 and Homer1A has been related also to synaptic impairment in Fragile X syndrome, schizophrenia and age-related memory impairment (Marton et al., 2015). Chiamulera and colleagues in a recent review (2017) proposed to targeting mGluR5 as a therapeutic intervention for inhibition of appetitive memory reconsolidation (Chiamulera et al., 2017).

In conclusion, metaplastic regulation of glutamate receptors and downstream cascade are potentially involved in a wide range of neurodegenerative and neuropsychiatric disorders, expanding the complexity of underlying mechanisms but also the possible targets that can be addressed for therapeutic purposes. However, despite the evidence presented above on metaplasticity involvement in substance and non-substance addiction as well as in memory processing, the influence of this high-order plasticity on these processes is far to be completely elucidated. Noteworthy, though in the last decade food addiction has received

increasing attention for the development of reliable therapeutic tools against obesity and obesity-related problems, the metaplasticity involvement in high palatable food memory consolidation, maintenance, reconsolidation or disruption remain to be fully investigated.

1.8 Aims

The present project was based on our previous experiments and literature evidence suggesting that it is possible to therapeutically address drug and non-drug addiction affecting the reconsolidation process of the maladaptive memories underlying these pathologies. Nevertheless, among the scientific community there are still conflicting opinions in considering high palatable food addiction as a distinct pathology sharing mechanisms of drug addiction, even though other eating disorders such as bulimia nervosa (BN), food craving (FC) or obesity have been already included in the recent DSM-V (Zou et al., 2017).

In light of these premises, this research has developed in two consecutive phases:

1) To demonstrate that food addiction memory can undergo reactivation and reconsolidation, combining a typical behavioural protocol of instrumental conditioning with a new two-component molecular assay;
2) To investigate whether the NMDA receptor blocker MK-801 given 24 h before retrieval could affect sucrose instrumental memory reconsolidation, following a protocol of administration known to induce metaplasticity (Buck et al., 2006).

For the first part, we followed the behavioural protocol previously used in our laboratory for the study of nicotine instrumental memory reconsolidation (Tedesco et al., 2014a). After instrumental training for sucrose self-administration, rats remained in their home cage for 14 days of abstinence, followed by operant memory retrieval (Ret group) or no-retrieval (No-Ret group) 24 hours later. The day after, memory reinstatement was evaluated with an extinction test. Along with the behavioural protocol, we decided to characterize the pattern of expression of two markers of drug memory reconsolidation, i.e. Zif268 and the rpS6P, 2 hours after memory reactivation. The former, Zif268, a

well-known molecular marker of memory reconsolidation, is an immediate early gene involved in *de-novo* protein synthesis after memory reactivation (Veyrac et al., 2014). Conversely, rpS6P, part of the 40S ribosomal subunit, is a relatively new marker of memory reconsolidation, and it is known as a downstream effectors of mTORC1 signaling cascade, important for both appetitive and aversive memories due to its role in protein synthesis locally in the active synapses (Barak et al., 2013; Tedesco et al., 2014b). The protein levels of these two molecular markers were evaluated in four brain regions that are involved in the reward mesocorticolimbic circuitry, i.e. nucleus accumbens, hippocampus, central nucleus of amygdala and basolateral complex of amygdala. Moreover, in amygdala we evaluated also the protein level of two glutamatergic receptors characterized for their important role in memory destabilization and restabilization, that is GluN2B for the former (Milton et al., 2013) and GluA1 for the latter process (Monfils et al., 2009).

For the second part, using the same behavioural protocol, we investigated whether the NMDA receptor blocker MK-801 was able to affect sucrose instrumental memory reconsolidation when given under a metaplasticity protocol. The PCP-like drug MK-801 has been already demonstrated to be effective in inhibiting memory reconsolidation in different paradigms when administrated close to retrieval session (Przybyslawski and Sara, 1997; Suzuki et al., 2004; Lee et al., 2006). However, only Buck and colleagues in 2006 demonstrated the metaplastic effect of MK-801. In fact, when administrated systemically in adult male rats, 4 mg/kg ip MK-801 was able to facilitate LTP induction in CA1-subiculum synapses in *ex-vivo* hippocampal slices 24 h after injection (Buck et al., 2006). Moreover, metaplastic effects has been shown also for another NMDARs blocker, i.e. ketamine. Ketamine, both intravenously and intraperitoneally, enhanced LTP in CA1 area of *ex-vivo* hippocampal slices 24 h after administration (Burgdorf et al., 2013; Graef et al., 2015). Moreover, 24 h post dosing, ketamine 10 mg/kg iv was able to induce antidepressant effects and to increase cell surface protein level of GluN2B and GluA1 in hippocampus and medial prefrontal cortex. In medial prefrontal cortex, ketamine increased also

GluN2B in a whole-cell lysate (Burgdorf et al., 2013). Zanos et al. (2016) finally, demonstrated that ketamine was able to increase GluA1 and GluA2 24 hours after treatment in mouse hippocampal, but not prefrontal, synaptoneurosomes (Zanos et al., 2016). These evidences indicate that NMDARs blockers possess a metaplastic action that eventually could have effects also on instrumental memory reconsolidation. Thus, following a behavioural protocol identical to the first part of the project, we conditioned rats to sucrose self-administration for 10 continuous days followed by 14 days of sucrose fasting. In this case, however, 24 hours before memory retrieval or no-retrieval we treated rats with either vehicle (saline solution) or MK-801 4 mg/kg ip. As before, after memory retrieval we evaluated memory reconsolidation with an extinction test 24 hours later.

For the molecular assessment, we analyzed the level of Zif268 and rpS6P in nucleus accumbens shell, central nucleus of amygdala and basolateral complex of amygdala as well as GluA1, GluN2B and mGluR5 in the nucleus accumbens and amygdala 2 hours after memory reactivation. The effects were investigated in all the experimental groups, i.e. Veh/Ret, Veh/No-Ret, MK-801/Ret and MK-801/No-Ret, in order to demonstrate that MK-801 administrated according to a metaplastic protocol can modulate key molecular markers for destabilization and restabilization, affecting reconsolidation of food instrumental memory.

All the behavioural phases of our experiments were performed in eight Skinner boxes equipped with two levers, one active on the right and one inactive on the left, a food magazine between the levers with the food dispenser behind the frontal wall, and an house light on the back panel of the box (Figure 8).

Figure 8: A Skinner box used for instrumental conditioning, retrieval and memory reconsolidation tests. It is possible to note, on the frontal wall of the box, the inactive lever on the left (a), the active lever on the right (b), the food dispenser between the two levers and the magazine behind the frontal wall (c), and the house light on the back wall of the cage (hidden, d). Moreover, it is visible the grid floor and two CS lamps that were not used during the experiments located over the levers. All the Skinner boxes were placed inside a sound-isolated cubicle equipped with a fan providing ventilation and a background white noise (e).

2. MATERIALS AND METHODS

2.1 Animals

One hundred and fifty-eight male Sprague-Dawley rats (Charles River, Italy) were housed in pairs in temperature and humidity-controlled environment (19-23°C, 60 ± 20 %) on a 12-h light/dark cycle, with light on at 7:30 pm. Rats were food restricted to maintain their body weight in the range of 250 ± 10 g (daily checked), and food (two to four pellets, 10-20 g/day) was made available after each experimental session. Water was available ad libitum, except during experimental sessions. Animals were trained or tested once daily during the dark phase of the light/dark cycle. All procedures were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986 and associated guidelines, and with EU Directive 2010/63/EU for animal experiments. All procedures were approved by the ethical committee (OPBA) of the University of Verona and by the Ministry of Health (authorization n. 271/2013-B). All efforts were made to minimize animal suffering and to keep the lowest number of animals used.

2.2 Apparatus

Rats were trained and tested in operant chambers (Coulbourn Instruments, Lehigh Valley, Whitehall, PA, USA) encased in sound-insulated cubicles equipped with ventilation fans (Ugo Basile, Comerio, Italy). Each chamber was equipped with two levers, an active (right) and an inactive lever (left), symmetrically oriented laterally to the food magazine, on the frontal panel. Levels were located 2 cm and food magazine 1 cm above the grid floor. A 2-W white house light was located 26 cm above the food magazine and provided ambient illumination during the entire session duration of food-shaping, retrieval and reinstatement phases, and for the entire session except for time-out (TO) periods during training phase. Right lever press produced the delivery of a 45-mg sucrose food pellet (Bilaney Consultants Ltd, UK) with a fixed-ratio 1 (FR1) schedule of reinforcement during training. Left lever presses did not have consequences. Lever presses and pellet deliveries were recorded, as well schedule parameters and data acquisition were controlled, by Med-PC software (Med Associates Inc., St Albans, Vermont, USA).

2.3 Phase #1

2.3.1 General Procedure

The experimental protocols were designed according to the following phases (Figure 9, panel A): Phase I) training to sucrose self-administration (S/A), Phase II) forced abstinence in home cage and, Phase III) memory Retrieval (Ret) or No-Retrieval (No-Ret). After these three subsequent phases, rats were divided in two groups: one group performed the reinstatement test in the training context 24 h and 7 d after Phase III, while animals in the second group were sacrificed for immunoblotting or immunohistochemical staining. During Phase III, rats were exposed to Ret or No-Ret session in the training context, i.e. Context A (AAA protocol) or in a novel context, i.e. Context B (ABA protocol).

2.3.2 Lever press shaping and training to sucrose self-administration

All rats were initially shaped to associate right lever presses with sucrose pellets as reinforcement. The schedule was an FR1 schedule, with the delivery of 45-mg sucrose food pellets, no delivery time-out during the procedures and sessions lasted up to 100 reinforcements or 120 min. Once the criterium of 100 reinforcements was reached, animals started training Phase I. During Phase I, right lever pressing delivered sucrose reinforcement with the same FR1, but with a 60-s time-out between two consecutive reinforcements, and the sessions lasted up to 12 reinforcements or 60 min. During time-out the right lever presses had no consequences, and the house light switched off. Left lever presses had no consequences for all the experimental period. After 10 continuous days of sucrose S/A training, rats remained 14 days in home cages for forced abstinence phase.

2.3.3 Behavioural analysis: retrieval procedure and reinstatement tests

After forced abstinence phase, rats were divided in two groups and exposed to a non-reinforced retrieval (Ret) or no-retrieval (No-Ret) session in the training context. During the Ret session, both the levers were presented and rats were allowed to press right active lever up to 20 times, with the house light on. Lever presses had no consequences. During the No-Ret session, no levers were presented and house light was off. During Ret or No-Ret session, animals spent a

similar amount of time in the training context (181±10 s; mean ± SEM). Two separate groups of rats were exposed to Ret or to No-Ret session in a novel context (Context B; operant conditioning chamber with 5-cm blank striped sheets on the walls and a 1-cm grid on the floor; Auber et al., 2014). Twenty-four hours after Ret or No-Ret all subjects were re-exposed for 60 min to the training context in the presence of levers, house light on and no time-out for a non-reinforced reinstatement test, to evaluate sucrose seeking behaviour. Lever presses had no consequences. Reinstatement test was replicated 7 d after retrieval Phase. All lever presses were recorded during Ret and reinstatement phases.

2.3.4 Molecular analysis: retrieval procedure and brain extraction

After forced abstinence phase, 4 separated groups of rats were exposed to Ret or No-Ret session, 2 groups in the training context and 2 groups in the novel context (Context B), as described in the previous section. Two hours after the first lever pressed during Ret or after the beginning of No-Ret session, all rats were sacrificed for Zif268 and rpS6P immunohistochemistry or GluN2B and GluA1 western blot investigations.

2.3.5 Immunohistochemistry

Rats were anesthetized with 350mg/kg/2 mL ip chloral hydrate (Fluka, Italy), then transcardially perfused with heparin 100 UI/L (Sigma–Aldrich, Milan, Italy) in saline solution and paraformaldehyde (PFA) 4 % in phosphate buffered saline solution (PBS). Brains were removed and post-fixed for 2 h at 4 °C into PFA 4 % in PBS, then washed 3 times with PBS and put in sucrose 30 % in PBS as cryoprotective for 48 h. Free-floating sections (40 μm) containing nucleus accumbens shell (NacS) (corresponding to a bregma +1.70 mm section from Paxinos and Watson, 1998); dorsal hippocampus (Hipp) (bregma -3.00 mm), central nucleus of amygdala (CeA) (bregma -1.88) and basolateral complex of the amygdala (BLA) (bregma -3.00 mm) were processed for Zif268 and rpS6P immunoreactivity. After washing in PBS, endogenous peroxidase was neutralized with hydrogen peroxide 0.75 % in PBS for 10 min. Sections were blocked with 0.5 % Horse Serum (HS; BioWhittaker-Lonza, Basel, Switzerland) + 0.5 % Triton

X-100 (Sigma-Aldrich, Milan, Italy) in PBS wash solution, and then incubated overnight at 4 °C with anti-Zif268 (1:1000, Santa Cruz, rabbit polyclonal) or anti-PSer235/236-rpS6 antibody (1:1000, Cell Signaling, rabbit polyclonal) in wash solution. After washes in wash solution, slices were incubated for 2 h at room temperature with anti-rabbit biotinylated antibody (1:1000, AmershamGE Healthcare Europe, Milan, Italy). Following washes in wash solution, and finally in PBS, tissue sections were visualized using VectaStain ABC kit (Vector Laboratories, Rome, Italy) and developed in DAB peroxidase substrate (Sigma-Aldrich, Milan, Italy) for 3-4 min. Sections were mounted on gelatin-coated slides, dehydrated and then closed with Entellan (Merck-Millipore, Darmstadt, Germany). The sections were acquired using a light transmission microscope (Axioscope 2 Zeiss, Zeiss). Six images for each region (1 for each hemisphere, 3 sections for each rat, that is 2 × 3 = 6 images/region/rat) were acquired by the connected video camera (Optikam B3) using a 10X objective (0.3 mm^2). Counts of the number of neurons positive to Zif268 and rpS6P were carried out using the NIH software 'Image-J' (www.rsbweb.nih.gov) (Caffino et al., 2016).

2.3.6 Western Blot

Rats were anesthetized with 350 mg/kg/2 mL ip chloral hydrate (Fluka, Italy), then brains were removed, and 1-mm slices containing amygdalae (bregma -3.00 mm) were dissected by using a 1-mm Coronal Brain Matrix (SouthPointe Surgical Supply, Florida, USA). Amygdalae were lysed in a tube containing 1 % sodium dodecyl sulfate (SDS). Total protein levels were quantified using the Pierce (Rockford, IL, USA) BCA (bicinchoninic acid) Protein Assay. Forty micrograms of proteins were resolved by electrophoresis on a 8 % SDS polyacrylamide gel and transferred onto nitrocellulose membranes using the transblot TURBO (Bio-Rad, Hercules, CA, USA). Blots were probed overnight at 4 °C with the polyclonal rabbit anti-GluA1 or the monoclonal mouse anti-GluN2B antibody (Thermo Scientific, Rockford, IL USA) diluted 1:1000 in 5 % milk. Immunodetection was performed with the secondary antibody anti-rabbit or anti-mouse (1:2000) (Amersham Biosciences, UK) conjugated to horseradish peroxidase. The reactive bands were detected using chemiluminescence

(ECLplus; Euroclone, Padova, Italy). Quantitative analysis was performed using the QuantityOne analysis software (Bio-Rad, Hercules, CA, USA) (Gerace et al., 2014).

2.3.7 Data Analysis

For the behavioural experiments, the number of active lever presses (ALP) and inactive lever presses (ILP) during reinstatement tests were compared to assess the effect of Ret versus No-Ret. Two separate two-way analyses of variance (ANOVAs) with the factors Condition and Test day were carried out on the total number of ALP or ILP/60 min in the reinstatement sessions.

For the immunohistochemistry experiments, intensity threshold, minimum and maximum cell size values were initially determined in an empirical fashion under blind conditions. The dependent variable for the immunohistochemistry experiments was the positive cell count/mm^2 for Zif268 or rpS6P. Immunohistochemistry and western blot data, as mean ± SEM percentages of the No-Ret rats, were analyzed by an unpaired Student's t-test. All analyses were performed using the GraphPad software package (Prism, version 4; GraphPad, San Diego, California, USA).

2.4 Phase #2

2.4.1 General Procedure

A schematic diagram of the protocol design is shown in Figure 14, panel A. The experimental protocols were designed according to the following phases: Phase I, training to sucrose pellets self-administration (S/A); Phase II, forced abstinence in home cage with M-K-801 or vehicle treatment 24 h before Phase III; and Phase III, memory Retrieval (Ret) or No- Retrieval (No-Ret) in the training context. After the three phases, rats were divided in two groups: one group performed the Reinstatement test in the training context 24 h after Phase III, while the animals in the second group were sacrificed for immunohistochemical staining 2 h after Phase III. Two separate groups of rats were treated with MK-801 or vehicle and sacrificed 24 h later for immunoblotting assays.

2.4.2 Lever press shaping and training to sucrose self-administration

All rats were initially shaped to associate right lever presses with sucrose pellets as reinforcement. The schedule was an FR1 schedule, with the delivery of 45-mg sucrose food pellet, without time-out between consecutive reinforcement, and session lasted up to 100 reinforcements or 120 min. Once the criterion of 100 reinforcements/session was reached, animals started training Phase I. During Phase I, right lever pressing delivered sucrose reinforcement with an FR1 schedule but with a 60-s time-out between consecutive reinforcements, and sessions lasted up to 12 reinforcements or 60 min. During time-out, right lever presses had no consequences and the house light switched off. Left lever presses had no consequences for all the experimental sessions. After 10 continuous days of sucrose S/A training, rats remained 14 days in home cage for forced abstinence phase.

2.4.3 Behavioural analysis: Retrieval procedure and Reinstatement tests

During the last day of forced abstinence, 24 h before Phase III, rats were divided in two groups, one treated with saline solution (vehicle) 1 mL/kg ip and one treated with MK-801 4 mg/kg/mL ip. The day after, both groups were further divided into subgroups exposed to unreinforced Ret or No-Ret session in the training context. The four resulting subgroups were: Vehicle/Ret, Vehicle/No-Ret, MK-801/Ret and MK-801/No-Ret. During the Ret session, both levers were presented, and rats were allowed to press right active lever up to 20 times, with house light on; levers had no consequences during Ret. During the No-Ret session, no levers were presented and house light was off. During Ret or No-Ret session, animals spent a similar amount of time in the training context (185 ± 10 s; mean ± SEM). Twenty-four hours after Ret or No-Ret session all subjects were re-exposed for 60 min to the training context in the presence of levers, house light on and no time-out for an unreinforced reinstatement test to evaluate sucrose seeking behaviour. Levers had no consequences during reinstatement. All lever presses were recorded during ret and reinstatement phases.

2.4.4 Molecular analysis: Retrieval procedure and brain extraction

For the molecular analysis, four separated groups of rats were treated with vehicle or MK-801 and exposed to Ret or No-Ret session as described in the previous section. Then, 2 h after the first lever emitted during Ret or 2 h after the beginning of No-Ret session, all rats were sacrificed for Zif268 or rpS6P immunohistochemistry.

2.4.5 Immunohistochemistry

Rats were anesthetized with 350 mg/kg/2 mL ip chloral hydrate (Fluka, Italy), then transcardially perfused with heparin 100 UI/L (Sigma–Aldrich, Milan, Italy) in saline solution and paraformaldehyde (PFA) 4 % in 1X phosphate buffered saline solution (PBS). Brains were removed and post-fixed for 2 h at 4 °C into PFA 4 % in PBS, then washed 3 times with PBS and left in sucrose 30 % in PBS for cryoprotection for 48 h. Free-floating sections (40 μm) containing NacS (corresponding to a bregma +1.70 mm section from Paxinos and Watson, 1998), CeA (bregma -1.88) and BLA (bregma -3.00 mm) were processed for Zif268 or rpS6P immunoreactivity. After washing in PBS, endogenous peroxidase was neutralized with hydrogen peroxide 0.75 % in PBS for 10 min. Sections were blocked with 0.5 % Horse Serum (HS; BioWhittaker-Lonza, Basel, Switzerland) + 0.5 % Triton X-100 (Sigma-Aldrich, Milan, Italy) in PBS wash solution, and then incubated overnight at 4 °C with anti-Zif268 (1:1000, Santa Cruz, rabbit polyclonal) or anti-PSer235/236-rpS6 antibody (1:1000, Cell Signaling, rabbit polyclonal) in wash solution. After washes in wash solution, slices were incubated for 2 h at room temperature with anti-rabbit biotinylated antibody (1:1000, Amersham GE Healthcare Europe, Milan, Italy). Following washes in wash solution, and finally in PBS, tissue sections were visualized using VectaStain ABC kit (Vector Laboratories, Rome, Italy) and developed in DAB peroxidase substrate (Sigma-Aldrich, Milan, Italy) for 3-4 min. Sections were mounted on gelatin-coated slides, dehydrated and then closed with Entellan (Merck-Millipore, Darmstadt, Germany). The sections were acquired using a light transmission microscope (Axioscope 2 Zeiss, Zeiss). Six images for each region (one for each hemisphere, 3 sections for each rat, that is 2 × 3 = 6 images/region/rat) were

acquired by the connected video camera (Optikam B3) using a 10X objective (0.3 mm2). Counts of the number of neurons positive to Zif268 and rpS6P were carried out using the NIH software 'Image-J' (www.rsbweb.nih.gov) (Caffino et al., 2016).

2.4.6 Pharmacological effects and western blot assays.

To elucidate the metaplastic effects of MK-801 on the level of glutamate receptors, two separated groups of 5 rats/group were treated with MK-801 4 mg/kg/mL ip or vehicle 1 mL/kg ip and 24 h later were anesthetized with 350 mg/kg/2 mL ip chloral hydrate (Fluka, Italy) and sacrificed. Then, brains were removed and 1-mm fresh tissue slices containing nuclei accumbens (+1.70 mm) and amygdalae (bregma -3.00 mm) were dissected by using a 1-mm Coronal Brain Matrix (SouthPointe Surgical Supply, Florida, USA). After dissection of brain areas, proteins of post-synaptic density and extra-synaptic fraction were analyzed as described in Caffino et al. (2017) with minor modifications. Briefly, nuclei accumbens and amygdalae were homogenized in a teflon-glass potter in cold 0.32 M sucrose buffer pH 7.4 containing 1 mM HEPES, 1 mM MgCl2, 1 mM $NaHCO_3$ and 0.1 mM PMSF, in presence of commercial cocktails of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich, Milan, Italy). Each homogenate was centrifuged at 800 g for 5 min; the obtained supernatant was then centrifuged at 13000 g for 15 min obtaining a pellet. This pellet was re-suspended in buffer containing 75 mM KCl and 1% Triton X-100 and centrifuged at 100000 g for 1 h. The resulting supernatant, referred as Triton X-100 soluble fraction (TSF, extra-synaptic fraction), was stored at -20°C; the pellet, referred as PSD or Triton X-100 insoluble fraction (TIF, post synaptic density), was homogenized in a glass–glass potter in 20 mM HEPES, protease and phosphatase inhibitors and stored at -20°C in presence of glycerol 30%. Total proteins have been measured in the TIF and TSF fractions according to the Bradford Protein Assay procedure (Bio-Rad, Milan, Italy), using bovine serum albumin as calibration standard. Equal amounts of proteins of the TIF fraction (8 μg) and of TSF fraction (15 μg) were run on a sodium dodecyl sulfate - 8% polyacrylamide gel under reducing conditions and then electrophoretically transferred onto nitrocellulose membranes

(GE Healthcare, Milan, Italy). Blots were blocked 1 h at room temperature with 10% non-fat dry milk in TBS + 0,1% Tween-20 buffer and then incubated with antibodies against the proteins of interest. The conditions of the primary antibodies were the following: anti-GluN2B (1:1000, Santa Cruz Biotechnology, USA), anti-GluA1 (1:1000, Neuromab, USA), anti-mGluR5 (1:1000, Millipore, Italy) and anti-β-Actin (1:10000, Sigma-Aldrich, Italy). Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories).

2.4.7 Data Analysis

For the behavioural experiment, the number of active lever presses (ALP) after 60-min reinstatement test from the four groups Veh/No-Ret, MK-801/No-Ret, Veh/Ret, MK-801/Ret was analyzed as dependent variable to assess the effect of drug treatment on retrieval condition. Two-way analysis of variance (ANOVA) with factors Treatment (Vehicle, MK-801) and Retrieval (Ret, No-Ret) was carried out on the total number of ALP after the reinstatement test. Two outlier subjects were discarded after outliers test. Fisher's LSD post-hoc tests were used to carry out only meaningful comparisons between Veh/No-Ret vs. MK-801/No-Ret and Veh/Ret vs. MK-801/Ret. For the immunohistochemistry experiments, intensity threshold, minimum and maximum cell size values were initially determined in an empirical fashion under blind conditions. The dependent variable for the immunohistochemistry experiments was the positive cell count/mm^2 for Zif268 or rpS6P. Two-way analyses of variance (ANOVAs) with the factors Treatment and Retrieval were carried out on mean ± SEM percentages of Zif268-positive cells/mm^2 and rpS6P-positive cells/mm^2 comparing the four different groups, with Vehicle/No-Ret as control group. Tukey's post-hoc tests were used to carry out all multiple comparisons. For the western blots assays, mean ± SEM percentages of Vehicle group were analyzed by an unpaired Student's t-test. All analyses were performed using the GraphPad software package (Prism, version 4; GraphPad, San Diego, California, USA).

3. RESULTS

3.1 Phase #1

The protocol of instrumental memory reconsolidation (Figure 9, panel A) showed no change of active lever presses/60 min after the reinstatement tests at 24 h (115.7 + 18.9 vs. 94.2 - 9.4, mean ± SEM) and at 7 d (56.1 + 12.7 vs 41.4 - 4.3, mean ± SEM) for the Ret condition vs. the No-Ret condition (NS, Two-way ANOVA for main effect for factor Condition $F[1,72] = 2.24$; NS for interaction factors Condition and Test Day $F[1,72] = 0.08$) (Figure 9, panel B).

Western blot analysis of total GluA1 protein level in the amygdala of rats exposed to Ret for instrumental memory showed a significantly higher level compared to rats under the No-Ret condition (+47 % GluA1/tubulin ratio vs. No-Ret; $p = 0.03$, Student's t-test), suggesting that our experimental condition induces the retrieval of sucrose instrumental memory in amygdala (Fig. 10, panel a).

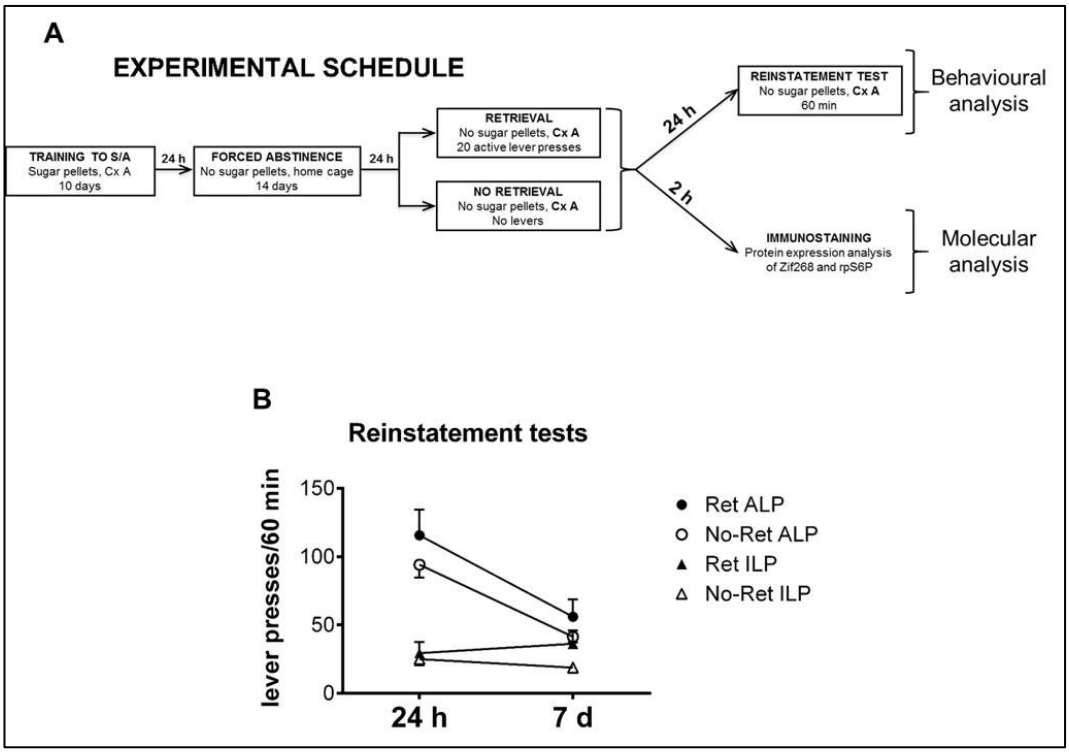

Figure 9. Effect of retrieval on reinstatement performance. (A) Schematic diagram of the experimental protocol and groups. Boxes represent the different procedures used at the different phases of the study. Arrow represents time progression between consecutive phases. Cx A = sucrose self-administration training

(conditioning) context. (B) Effect of Retrieval (Ret) or No-Retrieval (No-Ret) session in the training context on memory reconsolidation. Ordinate represents number of lever presses (circle = active, ALP; triangle = inactive, ILP) 24 h or 7 d after Ret (solid circle, solid triangle) or No-Ret (open circle, open triangle). Data are expressed as mean ± SEM. N = 18-20 rats/group.

Moreover, the analysis of total GluN2B protein level in the same brain area of rats exposed to Ret showed a significantly higher level compared to No-Ret (+43 % GluN2B/tubulin ratio vs. No-Ret; p = 0.004, Student's t-test), suggesting that our memory retrieval is followed by memory destabilization process taking place in amygdala (Fig. 10, panel b). The marker of memory reactivation Zif268 showed a significantly higher expression in the Ret vs. No-Ret condition in the NAcS, CeA and BLA but not in Hipp, respectively 28.2 + 6.2 ($p < 0.01$, Student's t-test), +30.7 + 5.4 ($p < 0.001$, Student's t-test), +68.2 + 7.6 ($p < 0.0001$, Student's t-test) and -11.0 + 7.7 (NS, Student's t-test) (Fig. 11). Correlational analysis showed that the number of Zif268 positive cells in CA1 area of hippocampus positively correlated with counts in BLA (r = 0.89, $p < 0.05$; Pearson's correlation) in the Ret but not in the No-Ret group (r = 0.71, p = 0.14; Pearson's correlation) (data not shown). As a further confirmation of reconsolidation process occurrence, we assessed the phosphorylation level of protein translation marker rpS6 in the same areas.

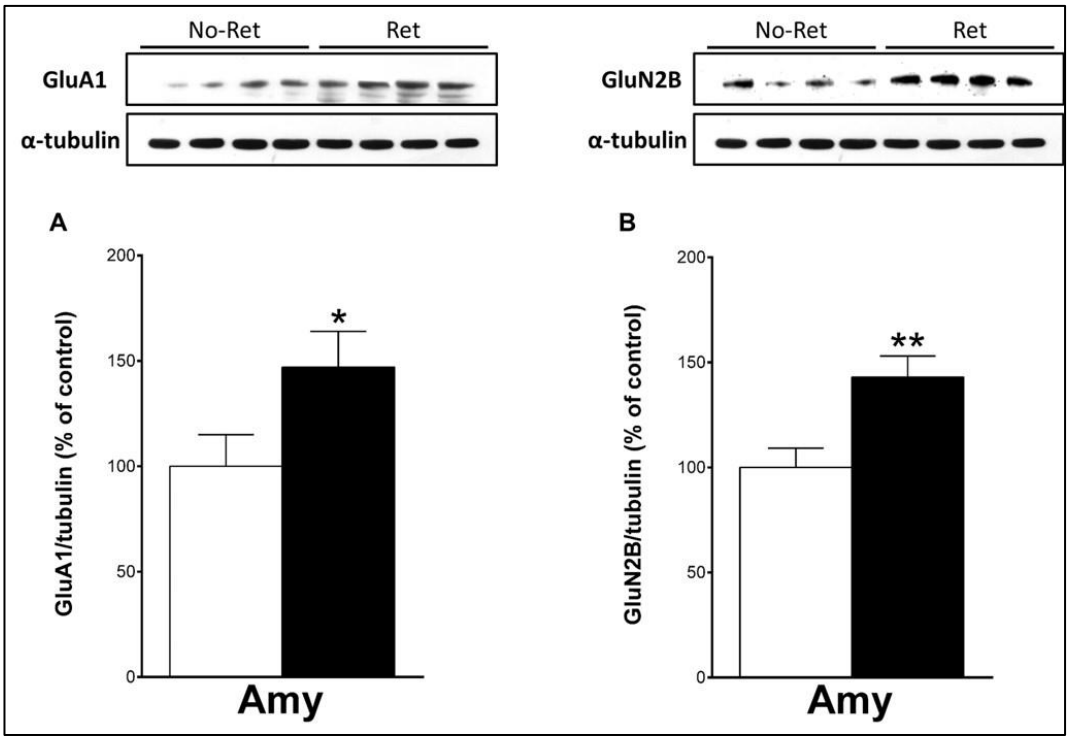

Figure 10. Effect of retrieval on total GluA1-AMPARs and total GluN2B-NMDARs in amygdala. In the upper part of the figure, representative immunoblots are shown for GluA1 (108 kDa, right) and GluN2B (180 kDa, left) proteins in the amygdala (Amy). (A-B) Quantification of the level of total GluA1-AMPARs (A) and total GluN2B-NMDARs (B) 2 h after No-Retrieval (No-Ret; open column) or Retrieval (Ret; solid column) of sucrose instrumental memory in rats. Data are shown as the mean + SEM and are expressed as a percentage of the No-Ret rats. N=7 - 8 rats/group. *$p < 0.05$; **$p < 0.01$ vs. No-Ret (unpaired Student's t-test).

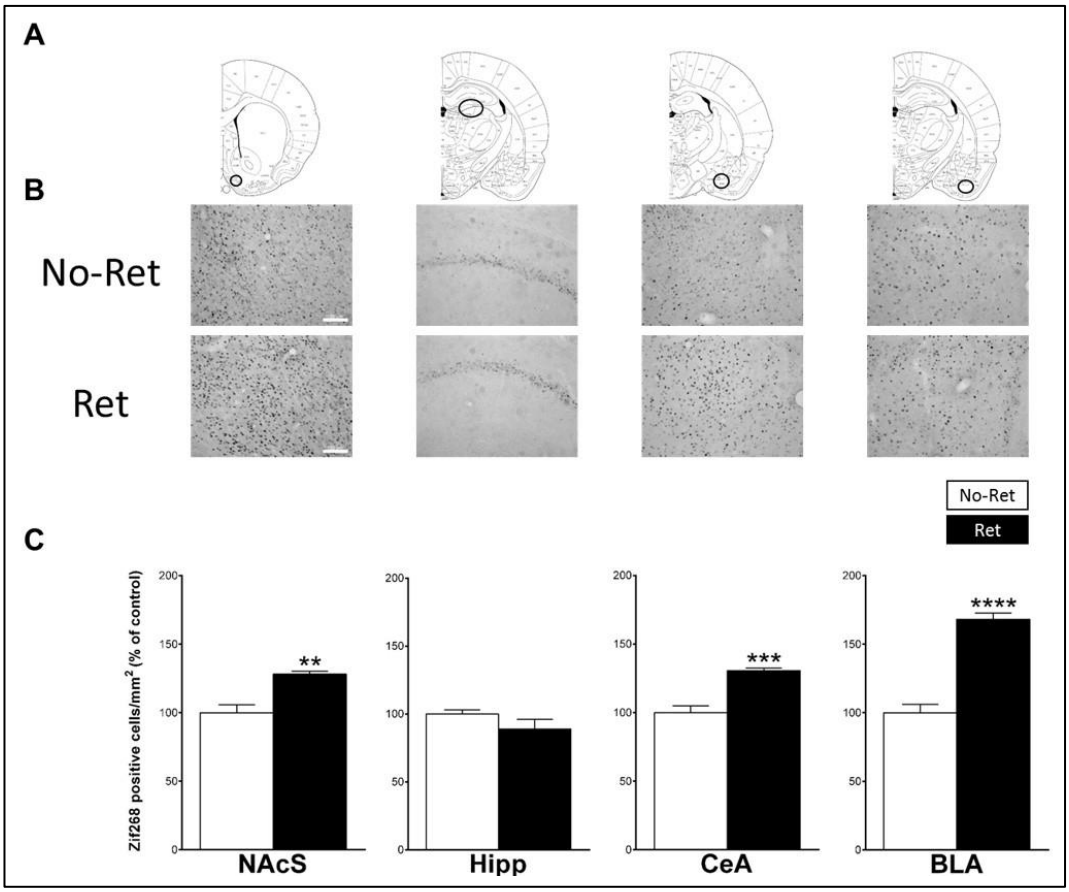

Figure 11. Immunohistochemistry assessment of Zif268 expression. (A) Representative images of brain areas of interest, with circles indicating the microscopic frame of the region under analysis. (B) Representative images of microscope sections of nucleus accumbens shell (NAcS), hippocampus (Hipp), central nucleus of the amygdala (CeA) and basolateral complex of the amygdala (BLA) 2 h after No-Retrieval (No-Ret; open columns) or Retrieval (Ret; solid columns) of sucrose instrumental memory in rats. Zeiss Axioskop 2, objective 10X. Scale bar, 100 μm. (C) Number of Zif268 expressing cells/mm^2 in NAcS, Hipp, CeA and BLA 2 h after No-Ret or Ret. Data are shown as mean + SEM and are expressed as a percentage of the No-Ret control group. Three adjacent sections, both hemispheres, N = 4-6 rats/group. **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ vs. No-Ret (unpaired Student's t-test).

Phosphorylated rpS6 was significantly higher in the Ret vs. No-Ret condition in NAcS, CeA but not in BLA, respectively +20.7 + 6.9 ($p < 0.05$, Student's t-test), +78.5 + 14.4 ($p < 0.001$, Student's t-test) and -3.6 + 7.4 (NS, Student's t-test). In

Hipp, phosphorylated rpS6 level was significantly reduced by the Ret exposure (-22.1 + 6.9, $p < 0.01$, Student's t-test) (Fig. 12).

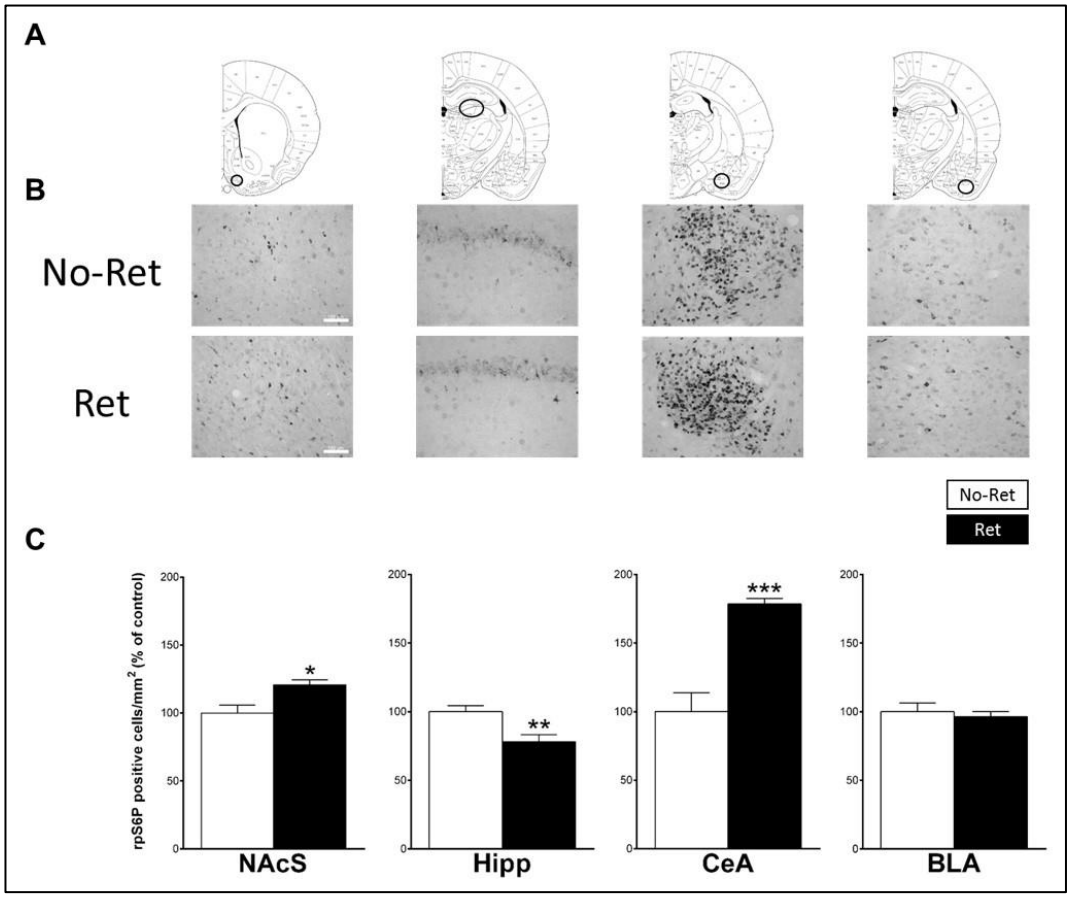

Figure 12. Immunohistochemistry assessment of rpS6P expression. (A) Representative images of brain areas of interest, with circles indicating the microscopic frame of the region under analysis. (B) Representative images of microscope sections of nucleus accumbens shell (NAcS), hippocampus (Hipp), central nucleus of the amygdala (CeA) and basolateral complex of the amygdala (BLA) 2 h after No-Retrieval (No-Ret; open columns) or Retrieval (Ret; solid columns) of sucrose instrumental memory in rats. Zeiss Axioskop 2, objective 10X. Scale bar, 100 μm. (C) Number of rpS6P expressing cells/mm^2 in NAcS, Hipp, CeA and BLA 2 h after No-Ret or Ret. Data are shown as mean + SEM and are expressed as a percentage of the No-Ret control group. Three adjacent sections, both hemispheres, N = 4-6 rats/group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. No-Ret (unpaired Student's t-test).

In order to test for experimental conditioning context effect on instrumental memory reconsolidation in our protocol, we assessed the levels of Zif268 and rpsS6P in a separate group of rats where the memory reactivation was performed in a novel context (i.e., a modified conditioning box – context B - provided with the same levers). Under this ABA protocol, Zif268 expression pattern in the NAcS and in the amygdala was similar to the original AAA protocol (NAcS: +29.0 + 5.2, $p < 0.001$, Student's t-test; CeA: +72.6 + 9.2 and BLA: +49.4 + 7.4, both at a significance level of $p < 0.0001$, Student's t-test). Interestingly, Zif268 was significantly increased in Hipp in Ret vs. No-Ret group, suggesting a context-related effect (+37.7 + 8.0; $p < 0.0001$, Student's t-test) (Fig. 13A), which however was not supported by any correlation between hippocampal CA1 and amygdalar nuclei. Phosphorylated rpS6 expression level, instead, was not changed in the NAcS, nor in the amygdalar nuclei CeA and BLA, and only slightly (-17.6 + 8.5, $p < 0.05$, Student's t-test) decreased in Hipp (Fig. 13B).

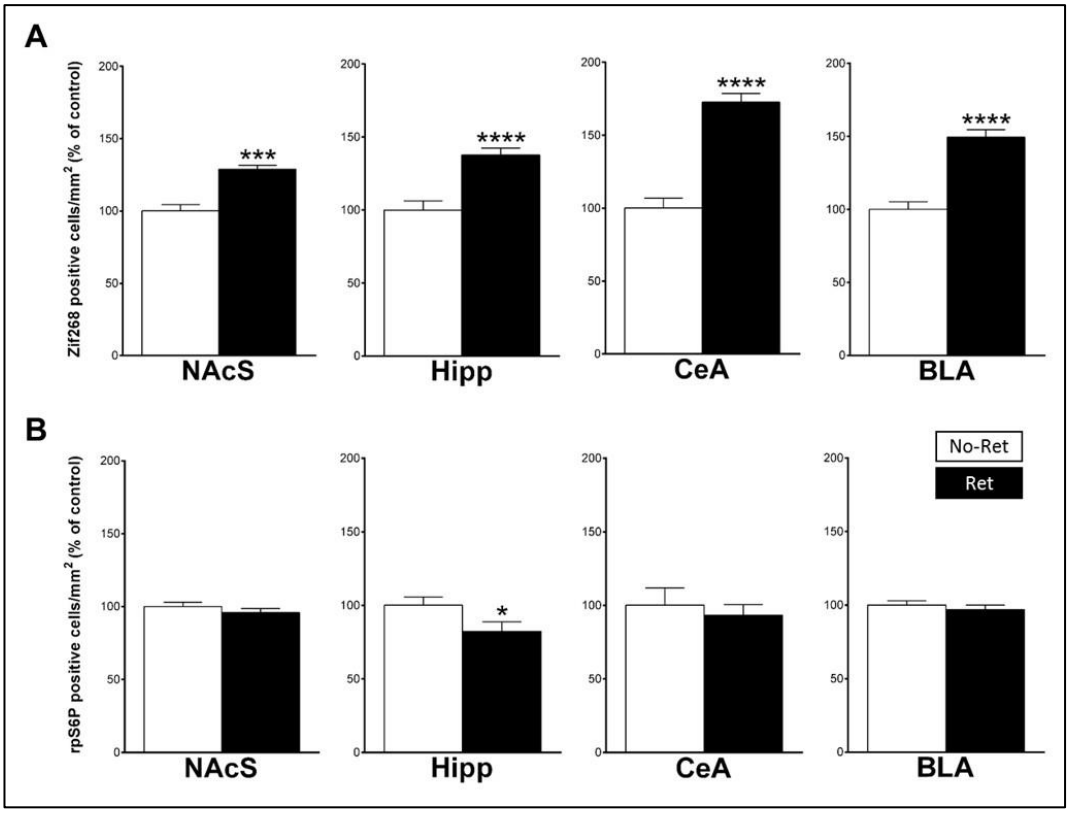

Figure 13. Immunohistochemistry assessment of Zif268 and rpS6P expression after retrieval in a novel context. (A) Number of Zif268 expressing cells/mm^2 in

NAcS, Hipp, CeA and BLA 2 h after No-Retrieval (No-Ret) or Retrieval (Ret) of sucrose instrumental memory. (B) Number of rpS6P expressing cells/mm^2 in NAcS, Hipp, CeA and BLA 2 h after No-Ret or Ret. Data are shown as mean + SEM and are expressed as a percentage of the No-Ret control group. Three adjacent sections, both hemispheres, N = 4 - 6 rats/group. *p < 0.05; ***p < 0.001; ****p < 0.0001 vs. No-Ret (unpaired Student's t-test).

In summary, the reactivation of instrumental memory for sucrose under our AAA condition allows for a retrieval process (increased GluA1) and a destabilization process (increased GluN2B) in amygdala, which is confirmed by increased Zif268 levels in amygdalar nuclei, and subsequent occurrence of reconsolidation process (rpS6P increasing in CeA, according to Barak et al., 2013). Although the control ABA condition showed a similar amygdalar Zif268 increase, a lack of rpS6P levels change did not however confirm the occurrence of the reconsolidation process.

Table II. Summary table of the direction of effects of memory retrieval in sucrose self-administration training context (AAA protocol) or in the no-conditioning context (ABA protocol) on Zif268 and rpS6P.

	AAA protocol		ABA protocol	
	Zif268	rpS6P	Zif268	rpS6P
NAcS	↑	↑	↑	0
Hipp	0	↓	↑	↓
CeA	↑	↑	↑	0
BLA	↑	0	↑	0

NAcS, nucleus accumbens shell; Hipp, dorsal hippocampus; CeA, central nucleus of the amygdala; BLA, basolateral complex of the amygdala. Symbols: ↑ = increase; ↓ = decrease; 0 = no change vs. No-Ret.

3.2 Phase #2

Two-way ANOVA on reinstatement test showed a significant main effect of factor Treatment [(F (1, 41) = 5.7; $p < 0.05$] but not of Retrieval [F (1, 41) = 0.09; NS] and of Treatment x Retrieval interaction [F (1, 41) = 1.5; NS]. Fisher's LSD post-hoc tests did not show significant differences between the two groups (94.3 ± 9.3 vs. 82.2 ± 11.8; NS) when comparing ALP/60 mins between Veh/No-Ret and MK-801/No-Ret at Reinstatement. On the other hand, Fisher's LSD post-hoc tests showed a significant decrease of active lever presses for MK-801/Ret compared to Veh/Ret group (103.9 ± 11.1 vs. 66.5 ± 7.7; $p < 0.05$) (Fig. 14B).

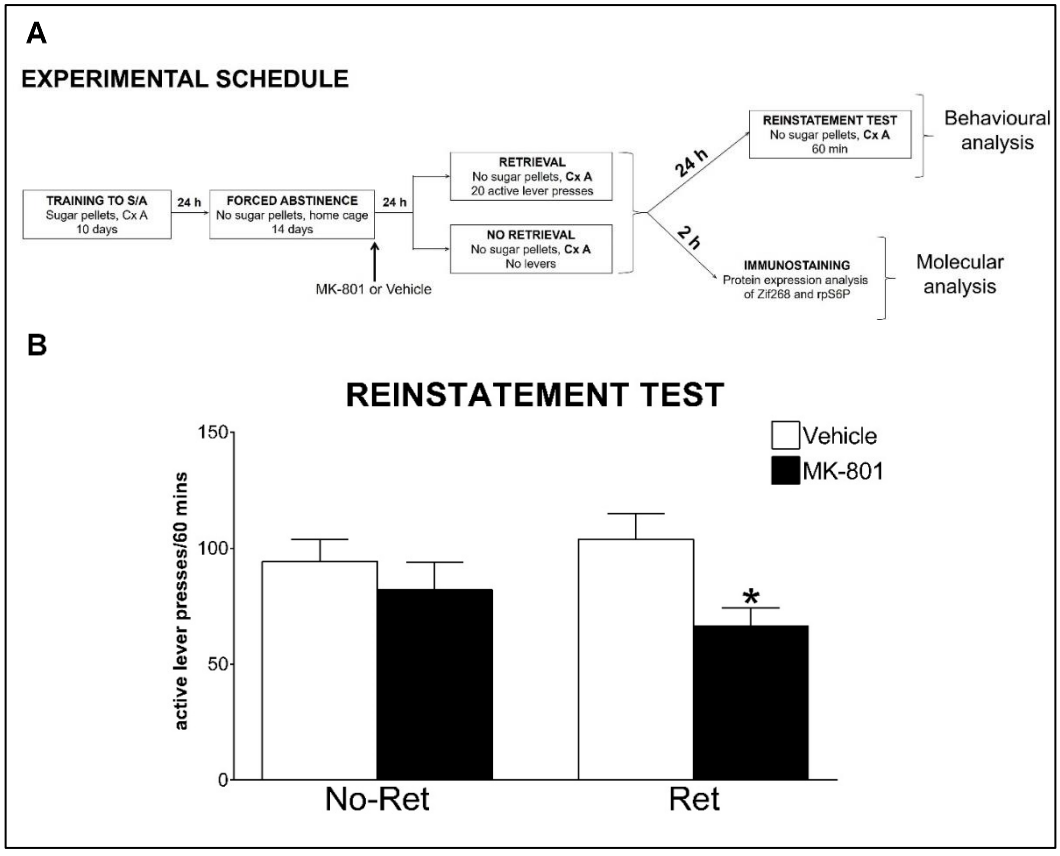

Figure 14. (A) Schematic diagram of the experimental protocol and groups. Boxes represent the different procedures used at the different phases of the study. Arrow represents time progression between consecutive phases. Cx A = sucrose self-administration training (conditioning) context. (B) Effect of Veh (open columns) or MK-801 (solid columns) treatment on Reinstatement performance when given 24 h before Retrieval (Ret) or not (No-Ret). Ordinate represents number of lever presses after Reinstatement test. Data are expressed as mean + SEM. N = 9-12

rats/Veh groups, N=11-13 rats/MK-801 groups. *$p < 0.05$, Fisher's LSD post-hoc tests.

The analysis of the expression of Zif268 in the NAcS showed a significant main effect of factor Retrieval [two-way ANOVA, ($F(1, 16) = 14.1$; $p < 0.01$] and of Treatment x Retrieval interaction [$F(1, 16) = 35.7$; $p < 0.0001$] but not of Treatment [$F(1, 16) = 0.6$; NS]. Comparing the different groups, Tukey's post-hoc tests showed a significant percentual increase of Zif268 expression in the MK-801/No-Ret compared to Veh/No-Ret (+37.1 ± 10.0; $p < 0.01$), a significant decrease for MK-801/Ret compared to Veh/Ret (-48.2 ± 10.2; $p < 0.01$) and a significant increase for Veh/Ret compared to Veh/No-Ret (+69.4 ± 10.6; $p < 0.0001$) (Fig. 15C). In the CeA, the analysis of Zif268 expression showed a significant main effect of factor Treatment [$F(1, 16) = 20.3$; $p < 0.001$] and of Treatment x Retrieval interaction [$F(1, 16) = 96.6$; $p < 0.0001$] but not of Retrieval [$F(1, 16) = 0.3$; NS]. Comparing the groups, Tukey's post-hoc tests showed a significant increase of Zif268 in MK-801/No-Ret compared to Veh/No-Ret (+85.4 ± 8.3; $p < 0.0001$) and a significant decrease of MK-801/Ret compared to Veh/Ret (-31.7 ± 8.5; $p < 0.01$). Moreover, Veh/Ret was significantly increased compared to Veh/No-Ret (+61.9 ± 8.8; $p < 0.0001$) and MK-801/Ret was significantly decreased compared to MK-801/No-Ret (-55.1 ± 8.0; $p < 0.0001$) (Fig. 15E). Similarly in the BLA, two-way ANOVA test showed a significant main effect of Treatment x Retrieval interaction [$F(1, 16) = 90.0$; $p < 0.0001$] but not of factor Treatment [$F(1, 16) = 0.8$; NS] nor of Retrieval [$F(1, 16) = 1.6$; NS]. Comparing the groups, Tukey's post-hoc tests showed a significant increase of Zif268 in the MK-801/No-Ret compared to Veh/No-Ret (+90.4 ± 12.9; $p < 0.0001$), and a significant decrease for MK-801/Ret compared to Veh/Ret (-74.9 ± 11.7; $p < 0.0001$). Moreover, Veh/Ret was significantly increased compared to Veh/No-Ret (+71.7 ± 13.0; $p < 0.001$) MK-801/Ret was significantly decreased compared to MK-801/No-Ret (-93.5 ± 11.6; $p < 0.0001$) (Fig.15G).

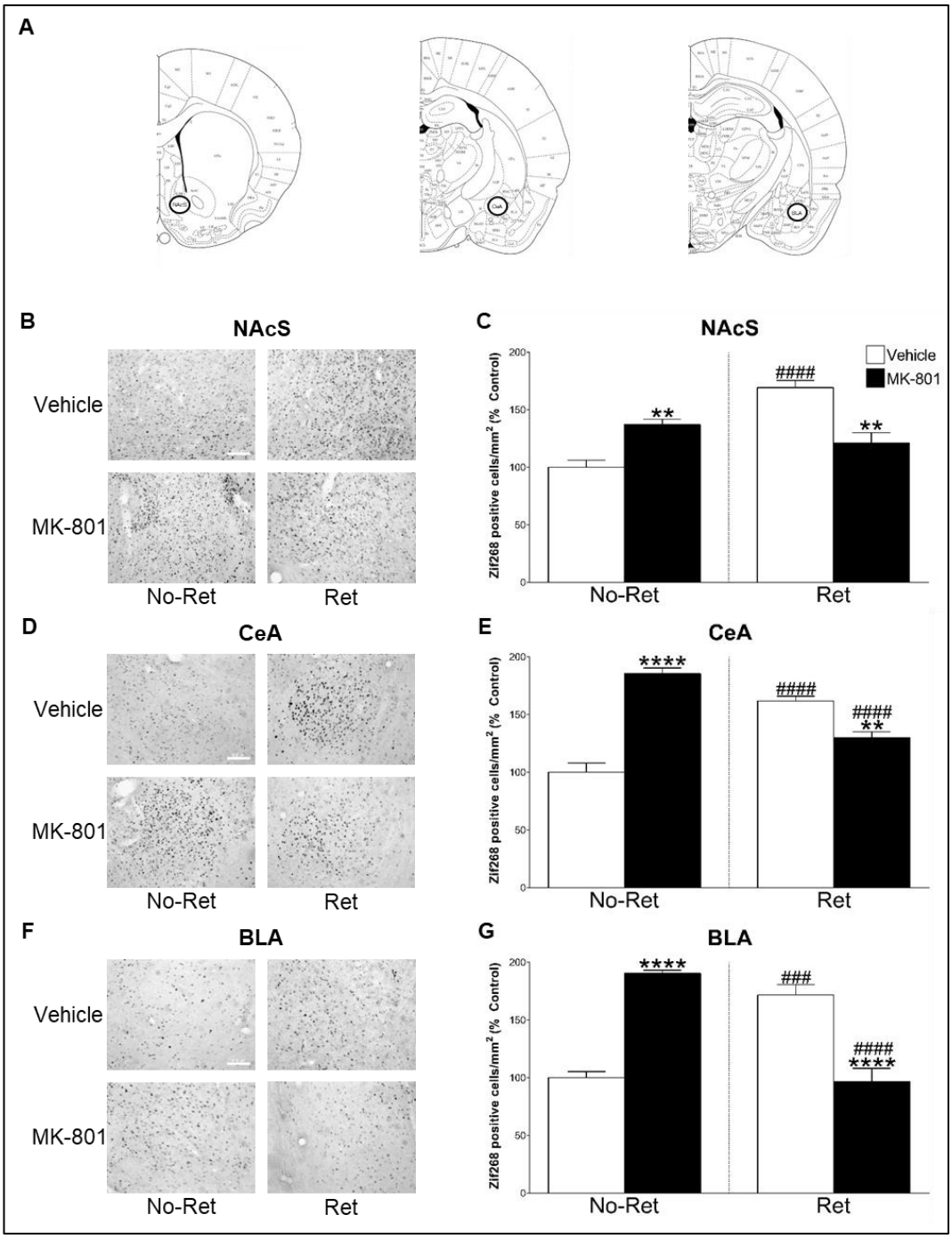

Figure 15. Immunohistochemistry assessment of Zif268 expression 24 h after vehicle or MK-801 treatment, and 2 h after Ret or No-Ret session. (A) Representative images of brain areas of interest, with circles indicating the microscopic frame of the region under analysis. (B, D, F) Representative images of microscope sections of nucleus accumbens shell (NAcS, B), central nucleus of the amygdala (CeA, D) and basolateral complex of the amygdala (BLA, F) 24 h after vehicle (open columns) or MK-801 (solid columns) and 2 h after No-

Retrieval (No-Ret) or Retrieval (Ret) session. Zeiss Axioskop 2, objective 10X. Scale bar, 100 μm. (C, E, G) Number of Zif268 positive cells/mm^2 in NAcS, CeA and BLA 24 h after vehicle or MK-801 and 2 h after No-Ret or Ret. Data are shown as mean + SEM percentual values of Vehicle/No-Ret. Three adjacent sections, both hemispheres, N = 4-6 rats/group. $^{**}p < 0.01$; $^{****}p < 0.0001$ between treatments (same Ret or No-Ret), $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$ vs. No-Ret within treatment, two-way ANOVA followed by Tukey's post-hoc tests.

To confirm the occurrence of memory reconsolidation process we assessed the phosphorylation level of protein translation marker rpS6 in the same areas. For the level of rpS6P in the NAcS, two-way ANOVA showed a significant main effect of Treatment x Retrieval interaction [F (1, 16) = 47.9; $p < 0.0001$] but not of factor Treatment [F (1, 16) = 0.01; NS] nor for Retrieval [(F (1, 16) = 1.4; NS]. Comparing the different conditions, Tukey's post-hoc tests showed a significant increase of rpS6P level in the MK-801/No-Ret compared to Veh/No-Ret (+31.3 ± 6.2; $p < 0.001$) and a significant decrease of MK-801/Ret compared to Veh/Ret (-30.3 ± 6.4; $p < 0.01$). Moreover, Veh/Ret was significantly increased compared to Veh/No-Ret (+25.5 ± 6.6; $p < 0.01$) and MK-801/Ret was significantly decreased compared to MK-801/No-Ret (-36.13 ± 6.0; $p < 0.0001$) (Fig. 16C). In the CeA, two-way ANOVA showed a significant main effect of factor Retrieval [F (1, 16) =29.1; $p < 0.0001$] and of Treatment x Retrieval interaction [F (1, 16) = 50.0; $p < 0.0001$] but not of Treatment [F (1, 16) = 2.9; NS]. Tukey's post-hoc tests showed a significant increase of rpS6P level in the MK-801/No-Ret compared to Veh/No-Ret (+48.4 ± 7.7; $p < 0.0001$), a significant decrease in the MK-801/Ret compared to Veh/Ret (-29.6 ± 7.9; $p < 0.01$) and a significant increase in the Veh/Ret compared to Veh/No-Ret (+68.7 ± 8.2; $p < 0.0001$) (Fig.16E). In BLA, two-way ANOVA showed no significant main effect of Treatment factor [F (1, 16) = 0.5; NS], of Retrieval [F (1, 16) = 0.2; NS] and of Treatment x Retrieval interaction [F (1, 16) = 1.3; NS]. Tukey's post-hoc tests showed no significant differences among the four different experimental groups (Fig. 16G).

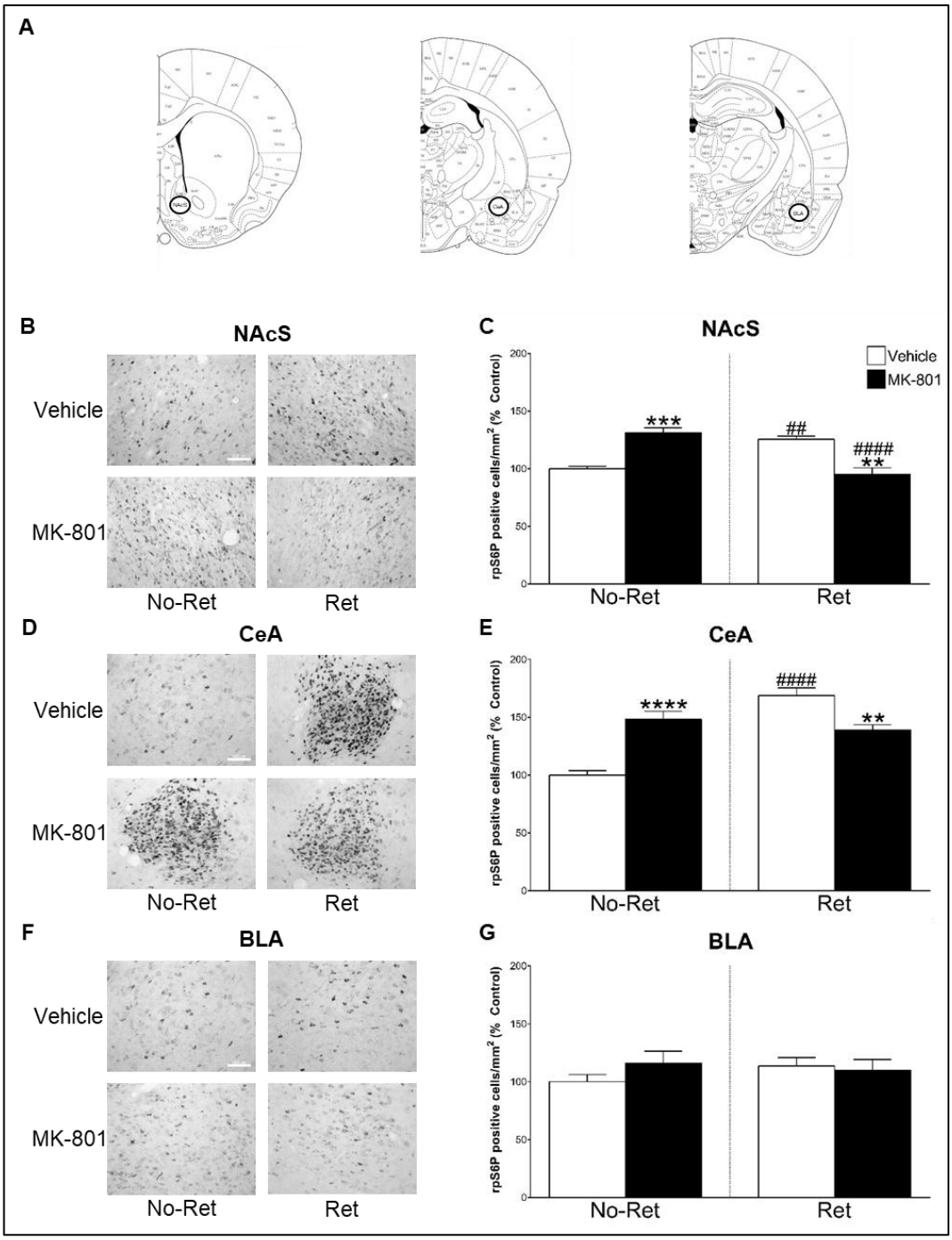

Figure 16. Immunohistochemistry assessment of rpS6P level 24 h after vehicle or MK-801 treatment and 2 h after Ret or No-Ret session. (A) Representative images of brain areas of interest, with circles indicating the microscopic frame of the region under analysis. (B, D, F) Representative images of microscope sections of nucleus accumbens shell (NAcS, B), central nucleus of the amygdala (CeA, D) and basolateral complex of the amygdala (BLA, F) 24 h after vehicle (open columns) or MK-801 (solid columns) and 2 h after No-Retrieval (No-Ret) or

Retrieval (Ret) session. Zeiss Axioskop 2, objective 10X. Scale bar, 100 μm. (C, E, G) Number of rpS6P positive cells/mm^2 in NAcS, CeA and BLA 24 h after vehicle or MK-801 and 2 h after No-Ret or Ret. Data are shown as mean + SEM and are expressed as a percentual values of Vehicle/No-Ret. Three adjacent sections, both hemispheres, N = 4-6 rats/group. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ between treatments (same Ret or No-Ret), $^{\#\#}p < 0.01$; $^{\#\#\#\#}p < 0.0001$ vs. No-Ret within treatment, two-way ANOVA followed by Tukey's post-hoc tests.

Western blot assays 24 h after MK-801 or Veh treatment in the post-synaptic density of NAc, the level of GluN2B and GluA1 after MK-801 was significantly increased compared to Veh (respectively +31.8 ± 4.9 and +47.4 ± 7.3; $p < 0.001$, Student's t-test). Moreover, the analysis of the extra-synaptic fraction showed that MK-801 significantly increased mGluR5 level compared to Veh (+47.1 ± 7.8; $p < 0.001$, Student's t-test) (Fig.17B). In the post-synaptic density of the amygdala, the level of GluN2B after MK-801 was significantly increased compared to Veh (40.2 ± 6.2; $p < 0.001$, Student's t-test), while the levels of GluA1 in the post-synaptic density and of mGluR5 in the extra-synaptic fraction did not show significant difference compared to vehicle (respectively +3.8 ± 3.3 and -5.3 ± 9.6; NS, Student t-test) (Fig. 17D).

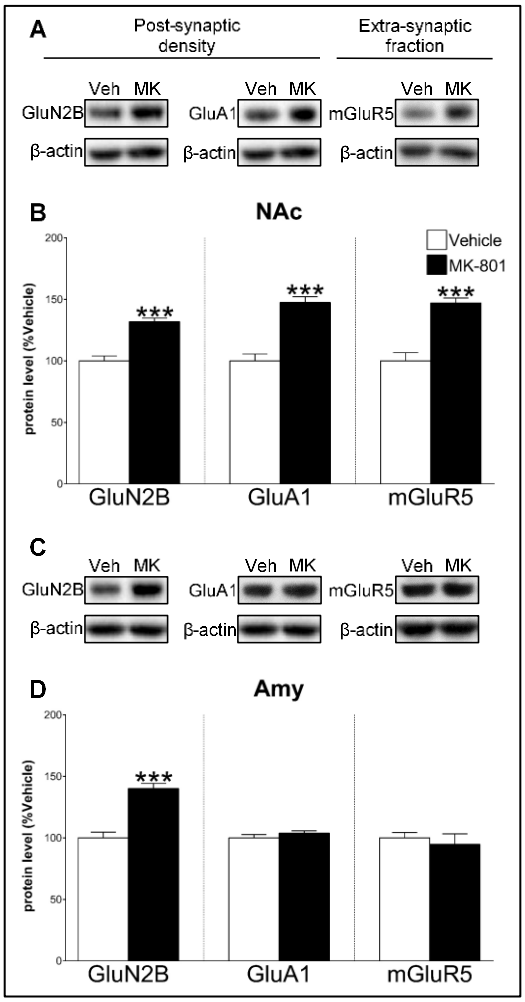

Figure 17. Effect of vehicle or MK-801 on GluN2B-containing NMDARs and GluA1-containing AMPARs in the post-synaptic density and on mGluR5 in the extra-synaptic fraction of nucleus accumbens and amygdala. (A, C) representative images of western blot bands with GluN2B (180 kDa, left) GluA1 (108 kDa, middle) and mGluR5 (130 kDa, right) compared to β-actin (43 kDa) as control. (B, D) quantification of GluN2B-NMDARs and GluA1-AMPARs level in the post-synaptic density and of mGluR5 in the extra-synaptic fraction 24 h after vehicle or MK-801 treatment in NAc (B) and amygdala (Amy) (D). Data are shown as the mean + SEM and are expressed as percentage of the vehicle. N=4-5 rats/group. ***$p < 0.001$, unpaired Student's t-test.

Table III. Summary table of the direction of effects of MK-801 or vehicle treatment on Zif268, rpS6P, GluN2B, GluA1 and mGluR5.

	Nucleus Accumbens Shell		Amygdala	
	Zif268			
	No-Ret	Ret	No-Ret	Ret
Vehicle	0	↑	0	↑
MK-801	↑	↑	↑	↓
	rpS6P			
Vehicle	0	↑	0*	↑*
MK-801	↑	↓	↑*	↑*

	Nucleus Accumbens	Amygdala
	GluN2B	
MK-801 vs Vehicle	↑	↑
	GluA1	
MK-801 vs Vehicle	↑	0
	mGluR5	
MK-801 vs Vehicle	↑	0

Symbols: ↑ = increase; ↓ = decrease; 0 = no change. * = in central nucleus of amygdala only.

4. DISCUSSION

In this project, we have investigated sucrose instrumental memory reconsolidation and whether this process could be affected applying a pharmacological treatment known to induce metaplasticity before the reactivation of the memory trace. The investigation was performed combining a behavioural protocol (developed and validated in our laboratory and shown to be able to evidentiate nicotine instrumental memory reconsolidation; Tedesco et al., 2014a) with a two-component molecular assay focusing on the protein level of Zif268 and rpS6P as markers of memory reactivation and reconsolidation in brain areas relevant for addiction, such as nucleus accumbens shell, central nucleus of amygdala and basolateral complex of amygdala. Moreover, the molecular assay was extended to GluN2B, GluA1 and also to mGluR5 in order to analyze the protein level of key glutamatergic receptors for both memory modulation and long-lasting synaptic remodeling in the nucleus accumbens and amygdala. The results can be summarized as follows: i) sucrose instrumental memory reconsolidation was demonstrated using the Zif268/rpS6P two-component molecular markers, even though behavioural test did not shown any significant results; ii) MK-801 intraperitoneal injection exerted a long-lasting metaplastic effect that, followed by memory reactivation, resulted in a significant inhibition of instrumental responding at the behavioural test iii) the molecular evidence supported the behavioural result, demonstrating that the levels Zif268, rpS6P as well as GluN2B, GluA1 and mGluR5 were affected by MK-801 before memory reactivation.

In the first part of the project, we showed that a protocol of instrumental memory reactivation induced memory retrieval and reconsolidation, as demonstrated by the increased expression of the transcription factor Zif268 and further confirmed by rpS6P, marker of mRNA translation and protein synthesis in active synapses. These molecular changes occurred in brain areas involved in appetitive memory retrieval and reconsolidation, i.e. nucleus accumbens shell, central nucleus of amygdala, and basolateral complex of amygdala. Molecular experiments performed to control for context effect showed that exposure to a

novel context during reactivation did not induce instrumental memory retrieval, as shown by the lack of rpS6P modulation despite Zif268 increase, and confirmed the specificity of increased rpS6P level in the nucleus accumbens shell and central nucleus of amygdala. The Zif268/rpS6P molecular markers appears to be reliable assays for instrumental retrieval and reconsolidation investigation, despite no behavioural changes during the reinstatement test. In fact, reconsolidation occurrence as an increased behavioural response has been rarely seen (Fuchs et al., 2009; Lasseter et al., 2011; Flavell and Lee, 2013; Tedesco et al., 2014a), and, as mentioned in the Introduction, reconsolidation occurrence is typically demonstrated through its inhibition (Lee, 2009).

The behavioural protocol used in the present study is similar to the one we previously used for retrieval and reconsolidation of nicotine self-administration. In that study, rats were trained to lever press for nicotine reinforcement without association to any discrete cue, as light or tone (Tedesco et al., 2014a). Similarly, in the present study we trained rats to lever press for sucrose pellets self-administration in a fixed number of consecutive trials, during which a limited amount of reinforcements per trials were available. After two weeks of forced sucrose fasting, reactivation of instrumental memory was induced by twenty non-reinforced lever presses, as previously shown for nicotine by Tedesco et al. (2014). However, differently from that study, we did not observe any significant increase of responding in retrieval vs. no-retrieval group at the reinstatement tests performed twenty-four hours and one week later. NMDARs antagonists such as MK-801, given after retrieval, have been shown to inhibit the reconsolidation of appetitive drug-associated memory in different Pavlovian conditioning paradigms in rats (Sorg, 2012). More importantly, as reported in paragraph 1.6.4, destabilization and reconsolidation of instrumental response for palatable food is possible – as confirmed by inhibited conditioned response for sucrose after blockade of protein synthesis (Mierzejewski et al., 2008) or NMDA receptors (Exton-McGuinness et al., 2014). In the latter, authors showed that instrumental memory reconsolidation occurred only when reactivated under a reinforced VR20 schedule of reinforcement, but not under a reinforced FR20 or non-reinforced (like our protocol) schedules. Moreover, Exton-McGuiness and Lee in 2015

confirmed instrumental memory reconsolidation also in weakly trained animals reactivated with a reinforced VR5, but not FR1 or non-reinforced session. Their findings were different from the earliest studies of Hernandez and Kelley (2004) and Mierzejewski et al. (2009) reporting no reconsolidation of instrumental memory after 15-day of sucrose S/A. However, both these studies used protein synthesis inhibitor, i.e. anisomycin the former and cycloheximide the latter, immediately after S/A. Considering that long-lasting training could have induced asymptotic learning that was reported as protein-synthesis independent (Rodriguez-Ortiz et al., 2005), it is conceivable to hypothesize that PSIs administrated after a training session are ineffective in disrupting memory reconsolidation. Moreover, in both the studies they used a training session including CS and US as memory retrieval session, thus lacking the novelty component that has been recently proposed as important for memory reactivation. In fact, the studies of from JLC Lee's Lab mentioned above suggested that the use of a reinforced variable-ratio (VR) schedule, but not of a Fixed-ratio (FR) or a non-reinforced schedule, owns those salient features of unpredictability that contribute to a prediction error signal and therefore to memory destabilization (Exton-McGuinness et al., 2015). Recent studies showed that a predictability change of the unconditioned stimulus appears to be associated with Pavlovian memory destabilization (Díaz-Mataix et al., 2013; Sevenster et al., 2013). In the case of Pavlovian memories, a VR schedule may provide a more reliable method of generating prediction error (Merlo et al., 2014; Piñeyro et al., 2014). According to the 'prediction error' hypothesis, Exton-McGuinness et al. (2015) suggested that our procedure in Tedesco et al. (2014a) might not induce reconsolidation of the instrumental component of memory. However, as they admitted in a sequent statement, it is not even possible to exclude that the non-reinforced FR-20 used in our experiments represent a sufficient change from training conditions able to trigger an error in prediction (Exton- McGuinness et al., 2014).

Another factor that should be considered is the role of the conditioning training context. Although there were no significant differences in responding at reinstatement tests between Ret and No-Ret groups under both AAA and ABA protocols (training, retrieval and tests in the conditioning context A for the former,

training in context A, retrieval in a novel context B and tests in context A for the latter), the expression level of Zif268 and rpS6P in the brain areas of our interest were different, suggesting the occurrence of memory reconsolidation under AAA but not ABA conditions. This context control experiment further suggests a potential role of the conditioning training context itself (i.e., Context A) as an occasion-setter, i.e. contributing but not sufficient *per sé* to memory retrieval (Bouton, 1993, 2004). In support of this hypothesis, Fuchs et al. have provided evidence about context-dependent reconsolidation of appetitive memories (Fuchs et al., 2009) and Flavell et al. (2011) demonstrated that the simple exposure to the training context was able to reactivate instrumental memory, as a following extinction session affected memory reconsolidation similarly to CS-exposure retrieval (Flavell et al., 2011).

The molecular assessment in key brain areas after instrumental memory reactivation was confirmed by the occurrence of processes such as retrieval and destabilization, as shown by increased expression in amygdala of GluA1 receptors for the former, and GluN2B receptors for the latter. GluN2B receptors have been shown to play a central role in the molecular processes that allow the destabilization of the memory trace (Finnie and Nader, 2012), whereas GluA1 receptors have been reported to be implicated in memory retrieval (Monfils et al., 2009; Clem and Huganir, 2010). Moreover, the antagonism of GluN2B blocks destabilization and the possibility for memory to undergo retrieval and reconsolidation (Ben Mamou et al., 2006; Milton et al., 2008a, 2012, 2013).

Transcription factor Zif268 has been widely used as a marker of memory reactivation and reconsolidation (Lee et al., 2004; Lee, 2008; Besnard et al., 2013), with its inhibition leading to reconsolidation blockade (Veyrac et al., 2014). In our study we observed a significantly higher expression level of Zif268 in basolateral complex of amygdala, as well as in other areas that are relevant for the reactivation of appetitive memory such as central nucleus of amygdala (Barak et al., 2013) and nucleus accumbens shell (Exton-McGuinness et al., 2015), supporting the reactivation of instrumental memory. Significant Zif268 increase in central amygdala but not in dorsal hippocampus further suggests a role of contextual information, without apparently involving a hippocampal-related

spatial information component. Interestingly, the role of central amygdala-related contextual value is confirmed by an increased level of rpS6P in this area, as originally showed by Barak et al. (2013) for alcohol memory reconsolidation. The role of the amygdala-dorsal hippocampus connections in memory reconsolidation has been investigated by Rita Fuchs's group showing that dorsal hippocampus is activated in response to amygdala activation, thus it does not play a necessary role by itself (Ramirez et al., 2009; Lasseter et al., 2011). Noteworthy, hippocampal Zif268 is significantly increased after retrieval vs. no-retrieval under ABA conditions, presumably due to the novelty features of the context B (Bozon et al., 2003).

Enhanced glutamatergic transmission through glutamate AMPA and NMDA receptors affects protein translation such as Akt and ERK1/2 (Gong et al., 2006): Akt activates phosphorylate mTOR and downstream rpS6 (Ferrari et al., 1991; Ruvinsky and Meyuhas, 2006), whereas ERK1/2 pathway induces rpS6 phosphorylation via p70S6K (Bessard et al., 2007) and p90S6K (Roux et al., 2007). This broad regulation of rpS6 phosphorylation makes it a sort of 'convergent' marker of those synaptic processes leading to rapid regulation of mRNA translation and *de novo* protein synthesis (for a review see Biever et al., 2015). The increased level of rpS6P in amygdala is a confirmation of the validity of this marker for the molecular assessment of memory reconsolidation (Barak et al., 2013; Tedesco et al., 2014b). Moreover, our study shows that an increased rpS6 phosphorylation in the nucleus accumbens shell indicates a specific increase of mRNA translation in brain nuclei relevant for appetitive memory. In the context-control condition ABA, rpS6P level was not different between retrieval and no-retrieval in central amygdala and nucleus accumbens shell, further confirming the specificity of our protocol of instrumental memory reactivation and reconsolidation under the AAA condition. On the other hand, we cannot currently explain rpS6P decrease in dorsal hippocampus; we exclude hippocampal rpS6P reduction due to different diurnal oscillation in the retrieval vs. the no-retrieval groups (Saraf et al., 2014).

As summarized in Table II, our findings suggest that the associated Zif268 and rpS6P expression assessment may be a molecular confirmation of transcription

(Zif268) and translation (rpS6P) activation respectively, with the latter specifically expressed by the training context condition AAA. We therefore proposed this dual-component molecular assessment as a reliable method, alternative to indirect (i.e., through inhibition) analysis of memory reconsolidation occurrence. In conclusion, this molecular approach confirmed that our protocol is able to reactivate instrumental appetitive memory and to trigger reconsolidation for sucrose. Together with our previous study on reconsolidation of nicotine instrumental responding (Tedesco et al., 2014a) and those from others (Exton-McGuinness et al., 2014; 2015), we suggest that also instrumental appetitive memory can undergo reactivation and reconsolidation.

In the second part of the project, we showed that MK-801 given 24 hours before instrumental memory reactivation (-24h MK-801/Retrieval) significantly inhibited the reinstatement of conditioned responding for sucrose. This effect was associated to a significant decrease of Zif268 in nucleus accumbens shell, central amygdala and basolateral amygdala, compared to Zif268 level in the same brain areas after vehicle followed by retrieval session. However, MK-801 *per sé* increased Zif268 level in the same brain areas after No-Retrieval compared to Vehicle/No-Retrieval group, similarly to the effect observed after the Vehicle/Retrieval session. Therefore, the behavioural inhibitory effect observed in the MK-801/Retrieval group could be specifically associated only in amygdala to a significant inhibition of increased Zif268 levels induced by either Vehicle/Retrieval or by MK-801/No-Retrieval.

The increased phosphorylation of rpS6P in the nucleus accumbens shell and central amygdala in the Vehicle/Retrieval group was reduced in the MK-801/Retrieval group. However, MK-801 *per sé* also increased phosphorylation of rpS6 in the same brain areas in the MK-801/No-Retrieval compared to Vehicle/No-Retrieval group. It therefore appears that the MK-801/Retrieval condition (associated to the behavioural inhibition of reinstatement) specifically inhibited increased rpS6 phosphorylation induced by either Vehicle/Retrieval or by MK-801/No-Retrieval only in the nucleus accumbens shell. On a separate

experiment, we have also shown that MK-801 increased GluN2B, GluA1 and mGluR5 expression level in nucleus accumbens and GluN2B in amygdala.

The behavioural inhibitory effect of -24h MK-801 on instrumental memory reconsolidation when given under a dosing protocol known to induce metaplastic effects (Buck et al., 2006; Zorumski and Izumi, 2012) appears to be necessarily associated to the procedure of the instrumental memory reactivation. This behavioural effect is specifically related to the inhibition of retrieval and reconsolidation markers Zif268 in both central and basolateral amygdala, and rpS6 phosphorylation in nucleus accumbens shell. The former molecular correlation is interestingly linked to -24h MK-801-induced increase of GluN2B synaptic levels in amygdala, a proposed metaplasticity marker of memory destabilization (Finnie and Nader, 2012). The effect on rpS6 phosphorylation in the nucleus accumbens shell is on the other hand associated to -24h MK-801-induced increase of GluN2B, GluA1 and mGluR5 levels in nucleus accumbens. These metaplastic molecular changes might have facilitated destabilization, respectively in amygdala (GluN2B) and/or in nucleus accumbens (GluN2B, GluA1 and mGluR5), allowing inhibition of the memory reconsolidation and then, inhibition of the reinstatement of instrumental responding.

We hypothesize that the metaplasticity mechanisms triggered by -24h MK-801 might have raised a direction change in the synaptic activation induced by the behavioural manipulation aimed to induce memory reactivation. The question however is: which is the process that after -24h MK-801-primed metaplastic condition and under memory retrieval allowed the inhibition of memory reconsolidation in the MK-801/Retrieval group? Or, as an alternative interpretation, is it possible that a process of extinction took place rather than reconsolidation inhibition? Indeed, it could be speculated that the facilitation of memory destabilization (as supported for instance by the metaplastic increase of GluN2B in amygdala) which should allow memory reconsolidation occurrence, it had on the other hand set in turn in the -24h MK-801/Retrieval group the conditions for a retrieval-contingent process of extinction rather than reconsolidation. The competition between reconsolidation and extinction processes during memory retrieval is a phenomenon that has been described for

reactivation sessions of intermediate length, which is neither short enough for reconsolidation nor too long for extinction, as reported for non-reinforced reactivation procedures (Flavell and Lee, 2013; Merlo et al., 2014). The metaplastic synaptic pattern changes induced by -24h MK-801 paired to the Retrieval, but not when paired to the No-Retrieval, condition may have shifted the balance between reconsolidation and extinction by favoring the acquisition of extinction as the predominant process, in this case as a new form of learning where responding was not associated to reinforcement delivery. It could be hypothesized that the parameters characterizing our retrieval session were close enough to the boundary conditions depicted in the paragraph 1.6.2, so that the metaplastic effects of MK-801 has been sufficient to shift the balance between reconsolidation and extinction occurrence towards the latter.

The decrease of Zif268 expression levels in amygdala in the -24h MK-801/Retrieval group may be interpreted as the possible molecular correlate of this shift to extinction. The transcription factor Zif268 has been widely used as a marker of memory reactivation and reconsolidation (Besnard et al., 2013; Lee, 2008; Lee et al., 2004). In the first part of the project, we showed that increased levels of Zif268 in amygdala and nucleus accumbens shell were correlated to memory reactivation and reconsolidation. Although Zif268 inhibition leads to reconsolidation blockade (Veyrac et al., 2014), however we cannot exclude that decreased Zif268 levels might also induced extinction facilitation. Trent et al. (2015) recently showed that the expression of immediate early genes (including Zif268) constrained extinction occurrence during the early phase of contextual memory reactivation; in fact knockdown of Zif268 levels during a short recall favored extinction occurrence rather than reconsolidation inhibition, whereas during a longer recall, changes in Zif268 had not effect. We could therefore speculate that with our reactivation parameters (similar to a short recall) a decrease of Zif268 levels in amygdala in the -24h MK-801/Retrieval group may have induced extinction.

Previous studies showed that increased rpS6 phosphorylation is correlated to memory reconsolidation (Barak et al., 2013; Tedesco et al., 2014b) and to reinforcing drug effect (Tedesco et al., 2013; Zanda et al., 2017). Due to the broad

regulation characterizing the phosphorylation of rpS6 (Ruvinsky and Meyuhas, 2006; Bessard et al., 2007; Roux et al., 2007), it cannot be possible to speculate which mechanism brought to reduction of the rpS6 phosphorylation even though a different balancing between signaling cascade predominance could be a potential cause.

An increase of GluN2B has been shown to facilitate destabilization (Ben Mamou et al., 2006; Milton et al., 2013; Wang et al., 2009). GluA1 instead has been demonstrated as important for memory retrieval (Clem and Huganir, 2010; Monfils et al., 2009) in a way dissociable from its role in destabilization (Milton et al., 2013), suggesting that these two processes may occur in parallel through two separate glutamatergic networks in amygdala (Lee and Flavell, 2014; Milton et al., 2013). Although there is a limited literature on Group I metabotropic glutamate receptors and memory reconsolidation (Gieros et al., 2012; Salinska, 2006), we have recently proposed that Group I subtype mGluR5 is involved in memory reconsolidation and mGluR5 antagonism may act as inhibitor based on the role played by the receptors in glutamatergic transmission modulation (Chiamulera et al., 2017). Moreover, glutamate receptors GluN2B, GluA1 and mGluR5 have been linked to different forms of metaplasticity, as reported in paragraph 1.7. Thus, in accordance to suggestions by Finnie and Nader (2012), the increased expression of glutamate receptors after -24h MK-801 may mediate the effects of increased glutamate release that occur when memory is reactivated. GluN2B levels appears to be important both in amygdala and nucleus accumbens not only for the mediation of metaplasticity and the regulation of memory destabilization, but also as an 'NMDARs dependency' factor that could explain the hypothetical "reconsolidation-to-extinction shift" suggested before, in accordance to the NMDARs-dependent reconsolidation-to-extinction transition in fear memory (Merlo et al., 2014).

In conclusion, our findings showed that NMDARs antagonism-induced metaplastic changes by MK-801 affected the retrieval of appetitive instrumental memories – either via reconsolidation inhibition or extinction facilitation – and the reinstatement of conditioned responding for sucrose. These data suggest that

pharmacological modulation of NMDARs given under a 'metaplastic dose-regimen' may be relevant for learning and memory at a behavioural level. However, the overall project has some limitations. First of all, as suggested above, we were not able to observe a significant increase in retrieval vs. no-retrieval group during reinstatement test in the first part of the project. In fact, even in presence of a trend of increase 24 hours after reactivation, the difference was not statistically significant. It should be taken into account, however, that only few studies were able to induce a significant increase in responding at test, and most of the studies demonstrated reconsolidation through its inhibition. Moreover, the rats of the no-retrieval control group were exposed to conditioning context without levers, in contrast to the retrieval group, where rats were exposed to conditioning context equipped with levers. Considering previous evidence by Fuchs et al. (2009), Flavell et al. (2011) and our control experiments (ABA protocol) depicting the conditioning context as an occasion-setter for memory reactivation, it could be consider to test our retrieval condition compared to a no-retrieval group were rats have been exposed only to their home-cage after saline treatment.

Moreover, we demonstrated memory reconsolidation through the Zif268/rpS6P molecular assay comparing the levels of the two reactivation and reconsolidation markers 2 hours after retrieval vs. no-retrieval sessions. To improve the reliability of this method it could be helpful to selectively block Zif268 increase and/or rpS6 phosphorylation in order to see whether these treatments could modulate reconsolidation occurrence. The same selective inhibition toward glutamate receptors could further highlight their fundamental role in memory reactivation and reconsolidation process.

Another important limitation of our project is the MK-801 time of treatment. In fact, usually memory is inhibited through the injection of NMDARs antagonists or PSIs closely to the reactivation session. Here, instead, we injected MK-801 24 hours before memory reactivation. The only evidence supporting a long-lasting effect of MK-801 is from Buck et al. (2006). However, in that study authors analyzed the facilitation of long-term potentiation in hippocampal synapses, while in our study we extended the interest to memory reconsolidation and to different

molecular markers related to reconsolidation and metaplasticity. Thus, a possible experiment could target memory reconsolidation with an MK-801 injection with dose and timing similar to previous studies (Lee and Everitt, 2008b; Tedesco et al., 2014a), in order to demonstrate that MK-801 is effective in inhibiting pure sucrose instrumental memory reconsolidation also in our experimental condition. Moreover, another aspect to be elucidated is the pharmacological effect of MK-801 on the metaplasticity and reconsolidation markers of our interest. We know that MK-801 half-life is 2 hours (Vezzani et al., 1989), so it is possible to exclude a direct effect of the drug on these markers. Nevertheless, a temporal characterization for the molecular mechanisms triggered by MK-801 and occurring during the 24 hours temporal period before memory retrieval is still lacking and should be investigated. Besides, further investigations should focalize the attention on synaptic and neuronal network activity changes. *In-vivo* electrophysiological recording (EEG) would allow studying in real-time the functional neuroanatomical connectivity during the procedure of instrumental memory reactivation and then during reinstatement test, without the methodological limitations of *ex-vivo* molecular post-retrieval assessment. Furthermore, *in-vivo* EEG recording could permit to extend our analysis also to the other phases of instrumental memory, such as conditioning and long-term memory tests, not replacing but supporting the *ex-vivo* evidence providing the possibility to identify electrophysiological correlates to the already known molecular markers of the different stages of memory processing. However, to assert with confidence that we are facing metaplastic effects on nucleus accumbens and amygdala – and possibly on other memory-related brain areas – after MK-801 systemic injection, an electrophysiological study on cultured brain slices, as performed in Buck et al. should still be considered for future experiments.

Finally, remain to be elucidated two more interesting aspects of long-lasting effects of MK-801: i) how NMDARs antagonism applied 24 hours before memory reactivation can shift processes from memory reconsolidation toward extinction occurrence and ii) whether this shift is long lasting. Identify a molecular marker or a signaling cascade specifically involved in this progression,

such as the increase of calcineurin in amygdala along with the shift from reconsolidation to extinction, as demonstrated for fear memory (Merlo et al., 2014), could be helpful to understand if the inhibition of conditioned responding is certainly due to extinction occurrence. Moreover, further analysis on Zif268 expression with Zif268 antisense oligonucleotide could be essential to understand whether, during sucrose instrumental memory reactivation, the expression of this transcription factor acts to prevent premature memory extinction or to induce memory reconsolidation, as suggested for fear memory by Trent et al. (2015). This knockout experiment could further clarify our hypothesis concerning to the transition from reconsolidation toward extinction. Lastly, testing for spontaneous recovery or memory reinstatement after sucrose re-exposure could help to understand if the effects of MK-801 and possibly other NMDARs antagonists suck as ketamine are long lasting and resistant to new exposure to unconditioned stimulus, such us sucrose – in our protocol – or drug of abuse, typically one of the main causes of relapse of addiction disorders.

4.1 Conclusion

Our findings suggest that sucrose instrumental memory reactivated with a short non-reinforced retrieval session can undergo reconsolidation, and this process can be blocked and possibly shifted to extinction occurrence with a MK-801 metaplastic treatment 24 hours before memory reactivation.

In conclusion, we suggest that a long-lasting metaplastic treatment with the NMDARs antagonist MK-801 combined 24 hours later with a short retrieval of sucrose instrumental memory may inhibit sucrose seeking, representing a potential new therapeutic intervention strategy addressing maladaptive appetitive memories.

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