ON THE TRANSITION FROM RECONSOLIDATION TO EXTINCTION OF CONTEXTUAL FEAR MEMORIES

by

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ABSTRACT

In this thesis we present three main studies, two regarding the transition of reconsolidation to extinction of contextual fear memories (Chapters II and III), and one on the mechanisms of reconsolidation under the synaptic tagging and capture (STC) perspective (Chapter IV). On the transition of reconsolidation to extinction, we observed a “null point” period between the parameters that induce reconsolidation and extinction of contextual fear memories, at which memory was insensitive to disruption by the amnesic agent MK-801, and some evidence for underlying STC mechanisms in the process of memory destabilization and reconsolidation. These findings reinforce and expand the hypothesis of a three-phase transition between reconsolidation and extinction of episodic-like memories and bring new insights on the different ways memory might be affected by reactivation and the mechanistic process involved.

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Chapter I

GENERAL

INTRODUCTION

1.1 Memory

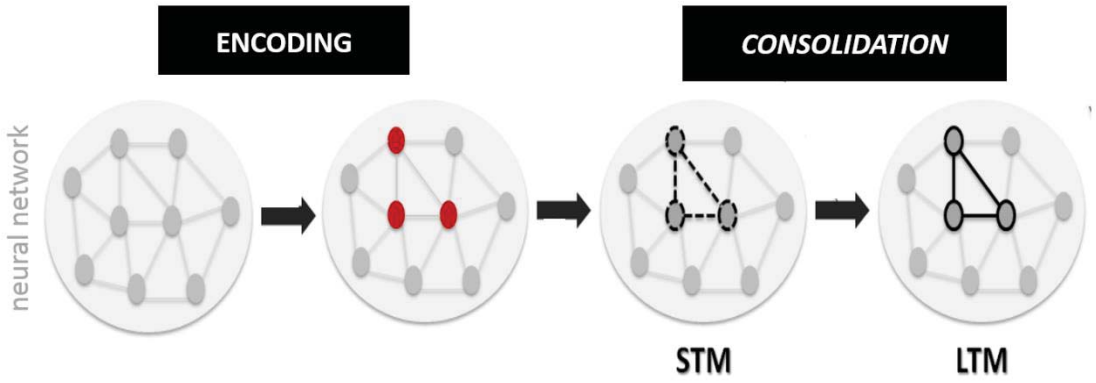
Memory can be defined as the storage of representative information that has been acquired through experience. Distinguishable memory systems operate in parallel to guide and support behaviour accordingly to different aspects and properties of an experience, as the content involved (Henke, 2010) and the persistence over time (Redondo and Morris, 2011).

Based on its content, memories can be differentiated into explicit (or declarative) and implicit (or non-declarative). Explicit memories are what people usually have in mind when referring to learning and memory process. It is associated with the encoding of events (episodic memory) and facts (semantic memory) (Templer and Hampton, 2013). Implicit memory refers to a broader range of skill-based information, that is expressed through performance and does not require conscious behaviour (Squire and Dede, 2015). It includes memories for skills and habits (procedural/instrumental memory) and simple classical conditioning (pavlovian memory).

Based on its persistence, memory can be differentiated into short-term, long-term, remote, and working-memory. Working memory is very short-living and last no longer than a few minutes (Constantinidis and Klingberg, 2016). Short-term memory (STM) is a more persistent learning that can be sustained for longer but will decay within a few hours (Vianna *et al.*, 2000). Memories that last for more than a day can be categorized as long-term memory (LTM). Long-term memories in rodents can last from days to weeks (Hardt, Nader and Nadel, 2013), depending on memory strength and properties, or even a whole life-time when stored in the form of remote memory (Frankland, Teixeira and Wang, 2007). Here we will be underlying the processes involved with episodic-like, long-term memories.

The establishment of a long-term memory is a process that involves an initial period of encoding, followed by a consolidation phase (Dudai, 2012). The encoding of a memory can be translated as the generation of a certain pattern in neural activity, in response to a given experience, that comprises representative information in the neural system (Zhou *et al.*, 2009). Initial modifications on the strength and weight of the synaptic connections in the network involved lead to the formation of short-time memory (STM). These modifications are then sustained and stabilized by the process known as cellular consolidation (Roger L. Redondo and Morris, 2011). The consolidation leads to the formation of long-term memory (LTM) and depends on the synthesis of plasticity-related proteins (Fig. 1.1).

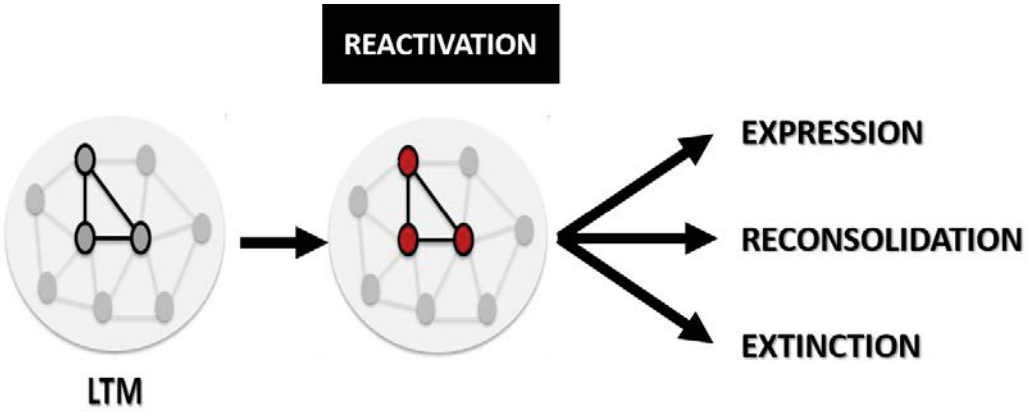
Figure 1.1



Schematic representation of the processes involvelved in the establishment of a memory.

After consolidation, similar situations may access the modified network and trigger memory reactivation. Memory reactivation, as the name says, is the process of bringing the memory to an active state again, which can initiate several other phenomena (Nader, 2015). The reactivation of the network can lead to the expression of the memory, which can be observed on behaviour, and then returns to inactivity until required again (Sevenster, Beckers and Kindt, 2012). Additionally, memory reactivation can trigger a process of destabilization/labilization followed by reconsolidation (Lee, Nader and Schiller, 2017), or even be subjected to active inhibition through extinction (de Carvalho Myskiw, Benetti and Izquierdo, 2013). (Fig 1.2). We will be focusing here on the process of reconsolidation and extinction, which are discussed next in more detail.

Figure 1.2



Schematic representation of possible post-reactivation memory processes

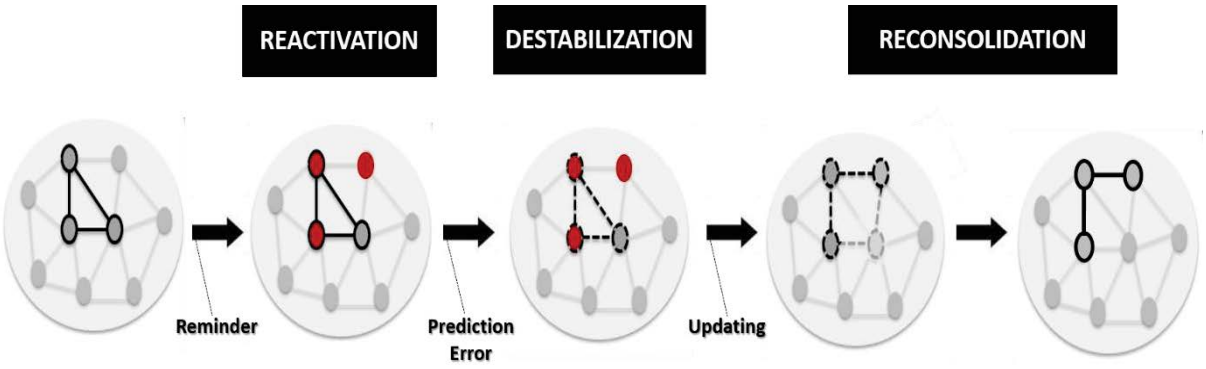
1.2 Reconsolidation

Memory reconsolidation is the process of restabilising a memory that has been destabilized/labilized after reactivation (Tronson and Taylor, 2007; Inda, Muravieva and Alberini, 2011; Nadel *et al.*, 2012; Nader, 2015). At first glance, it may seem a spurious process to enter memory into destabilization, since it will make memory susceptible to disruption and interferences and will require a full process of re-consolidation in order to persist. However, this process may actually serve an important adaptative role: it brings flexibility and malleability to memory (Lee, 2008, 2009, 2010). It is through this process that memories previously acquired and consolidated can incorporate modifications that may become necessary or available in the dynamic environment we live. These modifications include memory strengthening (Forcato, Fernandez and Pedreira, 2014), updating (Haubrich *et al.*, 2015), and maintenance of precision (De Oliveira Alvares *et al.*, 2013).

On the other side, inducing destabilization/reconsolidation of a memory may also bring the risk of inadvertent interreferences (Crestani *et al.*, 2015) that may lead to the disruption of relevant and important memories. Moreover, excessive modification of a memory could also negatively affect its accuracy over time, if erroneous or mistaken information are constantly incorporated to a memory (De Oliveira Alvares *et al.*, 2013). Therefore, there must be a mechanism that identifies the necessity or the potential for relevant modifications during memory reactivation and provides memory with adaptative malleability, without compromising quality (Lee, 2009). The generation of a prediction error has been proposed as the mechanism thorough which the system identifies the potential need of memory updating and initiates destabilization (Lee, 2009; Reichelt, Exton-McGuinness and Lee, 2013; Sinclair and Barense, 2018).

The prediction error would be triggered by a discrepancy in the expected and the actual outcome after memory reactivation, meaning the initial learning may have become outdated and require modifications. For example, if animals learn to associate the presentation of a cue with the delivery of a certain amount of food, and, when presented again to the same cue, they are presented with less food then expected, the discrepancy would generate a prediction error on the learned association, meaning, it might need updating (Flavell, Barber and Lee, 2011). By engaging memory destabilization, the prediction error would then allow for updating on the information and correction of the learned prediction (Fig. 1.3). On the other hand, if no discrepancy in the expected outcome is detected, and hence, the requirement of potential memory updating, memory would not undergo destabilization, and the risk of spurious modifications and the need of reconsolidation would be avoided (Díaz-Mataix et al. 2013; Exton-McGuinness et al. 2014).

Figure 1.3



The process of memory destabilization and reconsolidation. Schematic representation,

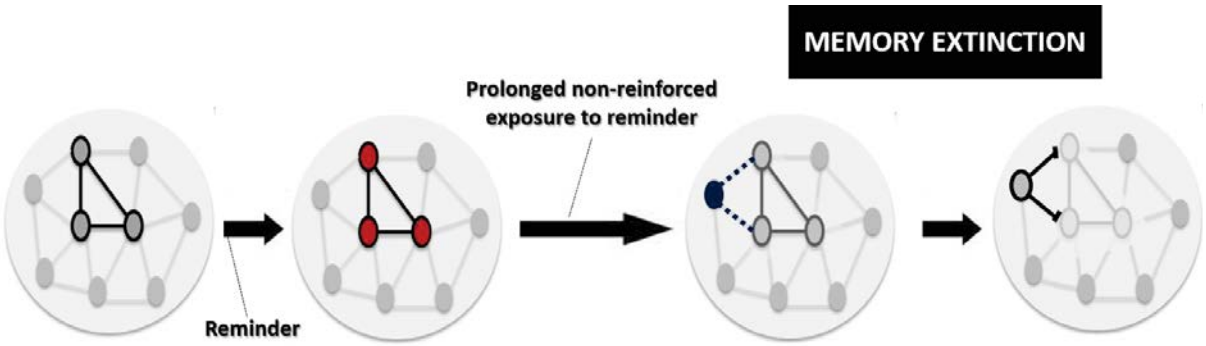
The destabilization of a memory, in addition to the detection of a prediction error, will also depend on the properties of the memory. Stronger (Wang, de Oliveira Alvares and Nader, 2009) and older memories (Frankland *et al.*, 2006), for example, will hold more defined expectations that will be less sensitive to occasional mismatching conditions, and therefore, increasingly resistant to interference and disruption. On the other hand, weak and recent memories will hold less detailed and less verified expectations that will more promptly respond to prediction error signals and undergo destabilization. This can be observed, for example, in the extend of reactivation under mismatching conditions that will be necessary to successfully induce destabilization and reconsolidation of a memory (Bustos, Maldonado and Molina, 2009). While a brief non-reinforced reexposure to a reminder may be enough to trigger destabilization of a weak or recent memory, older and stronger memories will require, under the same conditions, more extensive or intensive reactivation. Interestingly, though, if non-reinforced reactivation extends for too long, a different phenomenon called extinction may initiate, which will be discussed on the next session (Lee, Milton and Everitt, 2006).

In resume, the engagement of destabilization and reconsolidation will depend on a dynamic interaction between memory`s properties and characteristics (strength, age, etc), and the conditions present during memory`s reactivation (mismatching conditions, extend of reactivation, etc).

1.3 Extinction

Memory extinction is the process of actively supressing a memory through a new and opposing learning. As mentioned before, prolonged exposure to a reminder stimulus with the expected outcome continuously or repetitively omitted, may lead to a new learning where the given stimulus no more predicts the original outcome (Fig. 1.4). For example, the presentation of an auditory stimulus, that has been previously associated with the delivery of aversive footshock, will trigger the reactivation of the associated memory and the expectation of footshock delivery, which will be expressed on behaviour as a fear reaction (Lee, Milton and Everitt, 2006). If the footshock does not occur, though, and continue not occurring for a significant and extended amount of time it may generate a new learning where the same auditory stimulus becomes associated with an opposite outcome, that is: no-footshock. In order to express this new learning and avoid conflicting behaviour in future presentations of the auditory stimulus, the reactivation of the original memory will need to be actively inhibited, so that the stimulus does not trigger the previously associated fear response (Ehrlich *et al.*, 2009).

Figure 1.4



The process of memory extinction. Schematic representation.

This inhibitory learning has as characteristic the suppression, but not the disruption, of the original memory. The reason for the existence of this phenomenon may rely on the emotional salience of the original memory, since the study of extinction is commonly applied to aversive and strong experience, which are usually an indicative of important adaptative information (Olds, Lanska and Westerman, 2014). Therefore, in the event of occasional, but consistent, absence of an expected aversive outcome, it may be advantageous to adapt behaviour temporally, but not permanently. The maintenance of the original memory will allow its fast recovery and re-adaptation of behaviour in case the new learning is no further confirmed and/or shows to be potentially inaccurate (Dunsmoor *et al.*, 2015b).

The maintenance of the original memory can be assessed later through the phenomena of spontaneous recovery and renewal. Extinction learning usually is less strong and persistent then the original and strong learning. Therefore, with the passage of time the supressed memory usually is observed to spontaneously recover if no further extinction training or manipulations are applied (Bernal-Gamboa, Gamez and Nieto, 2017). Additionally, presentation of the conditioned stimulus outside the extinction context, or, the unconditioned stimulus with reduced intensity in the same context, may trigger fast recover of the aversive learning trough what is called renewal (Goode, Holloway-Erickson and Maren, 2017) and reinstatement (Augur *et al.*, 2016) respectively. Some behavioural and pharmacological manipulations able to enhance extinction may prolong the dominance of the extinction training over the original memory, but still, not definitively since memory itself is not disrupt in extinction, but only supressed. This can be assessed experimentally by the phenomena mentioned of spontaneous recovery, renewal and reinstatement (Fitzgerald, Seemann and Maren, 2014).

Memory extinction can also be identified and differentiated from actual disruption of the original learning by the effect of amnesic treatments on behavioural outcome. Since extinction involve a new learning that will inhibit the original one, amnesic treatments applied after extinction training are expected to lead to the maintenance of fear, not fear disruption (Fiorenza *et al.*, 2012). This is a useful observation in the study of extinction and memory processing, but also an important factor to be considered in the therapeutic approach of extinction when associated with pharmacological manipulations, as well as the use of reconsolidation. Since memory reconsolidation and extinction can be triggered by the same initial stimulus, that is, the presentation of a reminder without further reinforcement, it can be not very clear whether a given reactivation approach will lead to one or other, and pharmacological manipulations could actually lead to the oppose of fear attenuation. Therefore, for the therapeutic use of either reconsolidation or extinction in the treatment of pathological behaviours in the future, it is very important to have better understand of the boundary conditions that determine the progress of memory reactivation to one or other. Here is this thesis we will be analysing these conditions present during the transition of reconsolidation to extinction, in a contextual fear conditioning model.

1.4 Contextual Fear Conditioning

Contextual fear conditioning is a memory paradigm widely used in the literature as an animal model for aversive associative learning (Maren, Phan and Liberzon, 2013; Peters *et al.*, 2014; Izquierdo, Furini and Myskiw, 2016; Chaaya, Battle and Johnson, 2018). It usually involves one single session of training (or conditioning), where the animal learns to associate a given context (conditioning chamber) with aversive stimulation (footshock), and reexposure sessions, to induce memory reactivation and/or evaluate memory (test). Memory for the aversive event will be expressed later on as a fear reaction to the context, which can

be easily identified and quantified. In the face of unescapable treat, rodents express fear with a particular defensive behaviour known as *freezing*, which is characterized as a complete cessation of movement, except for that associated with respiration, in addition to a tense body posture and reduced heart rate (Blanchard, Griebel and Blanchard, 2001; Miki and Yoshimoto, 2010; Hagenaars, Oitzl and Roelofs, 2014). By measuring the percent of time animals express freezing behaviour, it is possible therefore to have a quantitative score of the animal`s memory for the previous aversive event, that can be easily and objectively assessed. For example on freezing behaviour, access: https://youtu.be/qFABuhoGr_E.

This paradigm offers a useful model for the study of psychobiological mechanisms in learning and memory. The one-trial conditioning and reexposure sessions assures a well-defined time frame for further manipulations of different memory phases and processes, and, its emotional content allows for the establishment of stable, reliable and long-lasting memories over time (Maren, Phan and Liberzon, 2013; Izquierdo, Furini and Myskiw, 2016; Chaaya, Battle and Johnson, 2018). Moreover, this associative aversive learning shares many properties with the development of fear memories in humans itself, and its better understanding may help the future development of more effective treatments and management of associated disorders, such as the post-traumatic stress disorder (PTSD), and other pathologies related to learning and memory in overall (Morellini, 2013; Nader, Hardt and Lanius, 2013). Finally, memory reconsolidation and extinction has been extensively studied in the contextual fear conditioning, making it a solid start point in the study of conditions and properties governing the transition of one process to the other (Gafford, Parsons and Helmstetter, 2011; Fiorenza *et al.*, 2012; de Carvalho Myskiw, Benetti and Izquierdo, 2013; De Oliveira Alvares *et al.*, 2013; Haubrich *et al.*, 2017).

Chapter II

ON THE TRANSITON OF

RECONSOLIDATION TO EXTINCTION

Part A

2.1 Introduction

Memory reactivation is an active process that can lead to different phenomena, such as reconsolidation (Nader 2009) and extinction (Giustino 2015), depending on several conditions. For instance, memory strength (Wang et al., 2009,), age (Frankland et al., 2006) and extent of reactivation (Suzuki et al., 2004) are all factors known to influence if and which path will be taken. A brief reactivation for example, tends to trigger a process of memory destabilization followed by reconsolidation, leading to memory maintenance. On the other hand, long reactivations without reinforcement tend to engage the long-term suppression of the memory, thorough a process known as extinction (Eisenberg et al., 2003; Duvarci et. al., 2006; Lee et al., 2006).

During both, reconsolidation and consolidation of extinction learning, the memory trace is transiently unstable and labile to modifications. Interestingly, however, it has been recently observed a "null-point" period on the transition of reconsolidation to extinction, during which memory does not seem to be sensitive to pharmacological manipulation, and neither processes appear to be actively engaged (Table 2.1).

In 2013, the first observation of the phenomenon was reported by Flavell and Lee in an appetitive memory setting. Lister Hooded rats were trained during five days to press a lever in order to receive food reward, which in turn, was associated with the presentation of a light stimulus. Three days after the completion of training, animals received a systemic injection of the amnesic agent MK-801(NMDA receptor antagonist), or its vehicle Saline, and were submitted to a reactivation session. During reactivation, lever-pressing resulted in the presentation of the light

conditioned stimulus (CS), but no food reward. After receiving 10, 30 or 50 unrewarded C.S presentations, animals were placed back into their homecages. Two days later, memory for the C.S-reward association was then assessed in a test session, accordingly to animal`s lever-pressing activity. Results have shown that MK-801 impaired memory reconsolidation that followed a brief reactivation session (10 C.S), as evidenced by reduced lever pressing at test. On the other hand, when animals were exposed to extensive unrewarded reactivation (50 C.S) MK-801 impaired memory extinction instead, preventing the decrease of lever presses observed on controls. Curiously though, MK-801 did not have any effect upon memory when reactivation was of intermediate duration (30 C.S).

Table 2. 1

Year	Authors	Memory Task	Reactivation Session	Drugs	Extend of Reactivation		
					Brief	Intermediary	**Long**
2013	**Flavell & Lee**	**Instrumental Appetitive Conditioning**	**10, 30 or 50** Presentations 0f Conditioned Stimulus (**C.S**)	**MK-801**[1] 0.1mg/kg, i.p. Pre-react.	✓ 10 C.S	∅ 30 C.S	✓ 50 C.S
2014	**Merlo et al.**	**Auditory Fear Conditioning**	**1, 4, 7 or 10** Presentations 0f Conditioned Stimulus (**C.S**)	**MK-801**[1] 0.1mg/kg, i.p Pre-react. **DCS**[2] 15mg/kg, i.p Pre-react.	✓ 1 C.S	∅ 4C.S	✓ 7 C.S 10 C.S
2015	**Alfei et al.**	**Contextual Fear Conditioning** (2 conditions)	**2, 6, 15 or 30** Minutes of Context Re Exposure.	**Midazolam**[3] 3 mg/kg, i.p. Post-react.	✓ 2min (cond. A) 6min (cond. B)	∅ 6 min (cond. A) 15 min (cond. B)	✓ 15 min (cond. A) 30 min (cond. B)

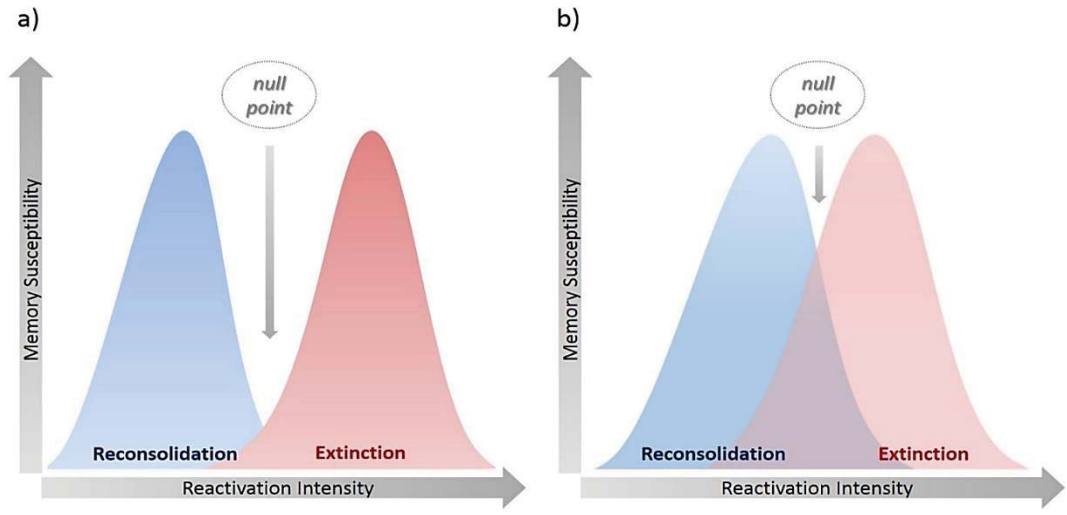
Published studies on the null-point of memory, until the year of 2015. [1]NMDAr antagonist. [2]NMDAr partial agonist. [3]Benzodiazepine. i.p. Intraperitoneal injection. ∅ No drug effect.

In 2014, Merlo and colleagues observed a similar phenomenon when studying auditory fear memories. Lister Hooded rats were conditioned to associate the presentation of a sound to the delivery of aversive footshock. One day later, animals were reexposed to either 1, 4, 7 or 10 presentations of the conditioned stimulus (sound), without aversive reinforcement. Thirty minutes before, animals received either MK-801 or the NMDA partial agonist D-cycloserine (DCS) systemically. The effect upon memory was then evaluated on the next day, accordingly to the level of fear expression (freezing) in response to the auditory stimulus. Again, MK-801 and, DCS, were able to significantly affect memory reconsolidation when reactivation was brief (1 C.S) and extinction when reactivation was more extensive (7 and 10 C.S), but had no effect when reactivation was of intermediate intensity (4 C.S).

Lastly, in 2015 Alfei and colleagues offered evidence that the discussed *null-point* was not restricted to appetitive and cued fear memories, nor to pharmacological manipulations of the NMDA receptor. First, Wistar rats were exposed to a specific context where they received foot-shock, either after 1 min (condition A) or 5 min (conditions B). Three days later, animals were re-exposed to the aversive context for 2, 6, 15 or 30 min without further reinforcement, in order to induce memory reactivation. Immediately after, animals received an intraperitoneal injection of the amnesic benzodiazepine Midazolam (3mg/kg), or its vehicle. Memory was then evaluated on the next day, accordingly to the level of fear expression (freezing) during further exposure to the context. Again, a similar *null-point* phenomenon was observed between the parameters that induced reconsolidation and extinction of memory on each of the training conditions. On condition A, Midazolam had a significant effect on memory when reactivation lasted for 2 or 15 min, but not 6 min. Similarly, on condition B memory was sensitive to Midazolam when reactivated for 6 or 30 min, but not 15 min. Moreover, the effects on memory were shown to be persistent and still evident when tested one week later.

These observations indicate that the *null-point* may represent an overall feature of associative memories, during which neither reconsolidation nor extinction seem to be in progress and, therefore, memory is not sensitive to pharmacological interference (Fig. 2.1a). However, it is also possible that a reactivation session of intermediate intensity would lead to diverse effects across subjects, if we consider the transitioning nature of the period and the natural variability in a population. Accordingly, we hypothesised an intermediate reactivation session could lead solely to memory reconsolidation in some individuals, while in others, memory extinction would have been already initiated. Subsequently, amnesic agents administered during this period would impair memory reconsolidation in part of the population, whereas affecting extinction learning in others instead. Hence, there would be no observable effect in the population as whole, not because memory itself is not undergoing either reconsolidation or extinction, but actually, because both processes can be found in a population during intermediate, transitioning conditions (Fig. 2.1b).

Figure 2.1



Proposed models for reconsolidation-extinction transition. a) Three-phase model. b) Two-fase model.

This might be expected to manifest as (a) a reduction in the correlation between freezing levels in the reexposure and test sessions in treated animals, (b) an effect of MK-801 when analyzing subpopulations determined by one or more factors (e.g., levels of freezing at context reexposure or test, or extent of within-session extinction during reexposure), or (c) increased variability in the MK-801-treated rats compared with saline-treated controls.

In order to further understand the nature and reinforce the generality of the null-point we aimed, first, to replicate the phenomenon on contextual fear memories, and later, to analyse in detail the pattern of behaviour encountered during the transitional phase to disambiguate its potential explanations, namely: (1) that the null point is a genuine effect and represents a phenomenon at the individual level or (2) that the null point is an artifact of variation in the transition point between reconsolidation and extinction at the population level.

2.2 Methods

Subjects: Subjects were 86 experimentally naïve adult male Lister Hooded rats, weighing 200–350 g at the start of the experiment, from Charles River (UK). Animals were housed in groups of four per cage, under a 12 h light–dark cycle (lights on at 07:00) and a 21°C temperature, with water and food provided ad libitum, apart from during the behavioural sessions. Cages contained aspen chip bedding, and environmental enrichment was available in the form of a Plexiglas tunnel. Experiments took place in a behavioural laboratory between 10:00 and 14:00. At

the end of the experiment, animals were humanely killed via a rising concentration of CO2; death was confirmed by cessation of heartbeat. All procedures were approved by a local ethical review committee and conducted in accordance to the United Kingdom Animals (Scientific Procedures) Act 1986, Amendment Regulations 2012 (PPL 70/7662).

Behavioural apparatus: The conditioning chambers (MedAssociates) consisted of two identical illuminated boxes (25 cm × 32 cm × 25.5 cm), placed within sound-attenuating chambers. The box walls were constructed of steel, except by the ceiling and front wall, which were made of Perspex. The grid floor consisted of 19 stainless steel rods (4.8 mm diameter; 1.6 mm centre to centre), connected to a shock generator and scrambler (MedAssociates). Infrared video cameras were mounted on the ceiling of the chambers (Viewpoint Life Sciences) and used to record behaviour.

Contextual fear conditioning: The behavioural procedure was adapted from de Oliveira Alvares et al. (2012) and consisted of a training, a reactivation, and a test session. During training, rats were placed individually in the conditioning chambers. After 3 min of free exploration, animals received 2 footshocks (0.7 mA, 1.5 sec) separated by a 30-sec interval, and after 1 min, were placed back into their homecages (training session). Two days later, animals were reexposed to the same context for 3, 5, 10, 20, or 30 min (reactivation session). One day later, animals were once again exposed to the conditioned context, for 3 min, in order to access memory expression (test session). No footshock was applied at either reexposure or test sessions. The aversive behaviour (freezing), in response to the conditioning context, was automatically quantified during all sessions with a videotracking software (Viewpoint Life Sciences) and used as memory index (Lee and Hynds 2013; Song et al. 2016).

Drugs: MK-801 (Sigma-Aldrich) was diluted in sterile saline (0.1 mg/ml) and injected intraperitoneally (1 ml/kg) 30 min before the reactivation session (Flavell & Lee., 2013, Merlo et. al., 2014). Injections of MK-801 or vehicle were randomly allocated between animals accordingly to order generated with List Randomizer (https://www.random.org/lists).

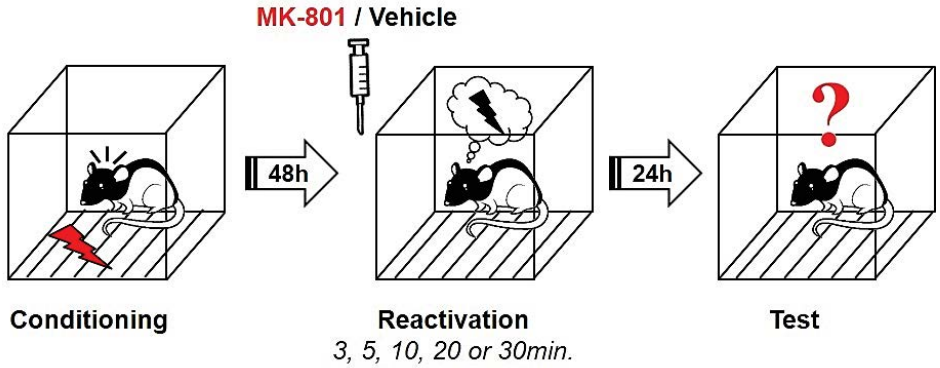
Statistics: Data were analysed in SPSS (IBM Corp, 2015). Single between-group comparisons (vehicle x MK-801), were performed on each reactivation condition with one-way ANOVA. Repeated-measures ANOVA was performed for within-group comparisons (reactivation x test). Significance was set at $p < 0.05$ and data are presented as mean + SEM. As an estimate of effect size, n_p^2 was used. Animals freezing more than 95% or less than 5% during reactivation, were excluded from analysis (2 and 0 animals, respectively). The rationale for this was that asymptotic learning appears to result in a resistance to memory destabilization (Rodriguez Ortiz et al. 2005, 2008; Lee 2010), and so rats that froze at near maximal levels during context reexposure would be unlikely to undergo reconsolidation regardless of reexposure duration. Similarly, animals that do not learn at all would not be suited to detect reconsolidation or extinction impairments, and so a criterion of >5% freezing was also imposed, although this did not result in the exclusion of any animals.

2.3 Results

In order to confirm the existence of the null point in the reactivation of contextual fear memories, Lister-Hooded rats were subjected to a contextual fear conditioning (CFC) paradigm, consisting of training on day 1, reactivation on day 3 and test on day 4 (Fig. 2.2).

The duration of the reactivation session varied across experiments and lasted for 3, 5, 10, 20, or 30 min. The NMDA receptor antagonist MK-801 was injected intraperitoneally (0.1 mg/kg) 30 min before reactivation session. MK-801 is a well-known amnestic agent that leads to distinctive outcomes on behaviour when reconsolidation or extinction are affected (Lee et al. 2006; Flavell & Lee 2013; Merlo et al. 2014). The aversive response (freezing) was automatically recorded during all sessions and used as an index of fear memory.

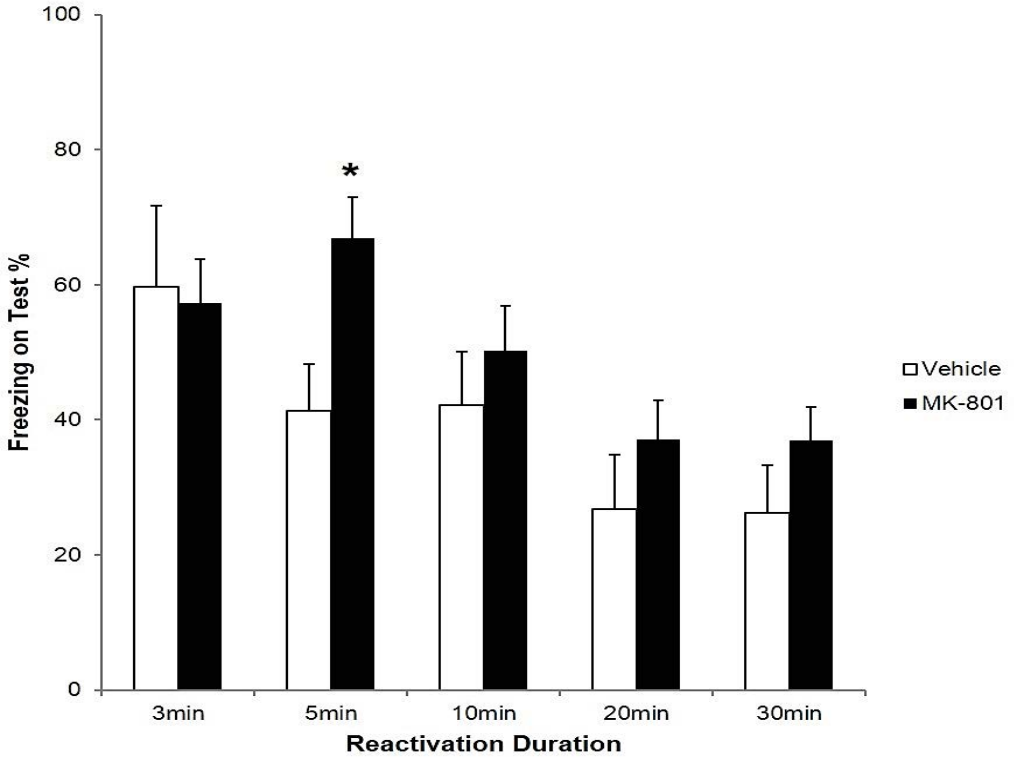
Figure 2.2



Schematic representation of experimental design applied.

Figure 2.3 shows expression of fear memory during the test for each reactivation condition. There was no significant effect of MK-801 with the intermediate, 10 min ($F_{1,15}$ = 0.53, p = 0.478; n = 8 per group) and 20 min ($F_{1,15}$ = 1.79, p = 0.203; n = 8 per group), conditions. However, we did not observe any effect either with the 3-min ($F_{1,23}$ = 0.09, p = 0.770; n = 12 per group) and the 30-min ($F_{1,14}$ = 1.63, p = 0.225; n = 7-8 per group) sessions. Moreover, there was a significant effect of MK-801 with the 5-min condition ($F_{1,14}$ = 7.7, p = 0.016; η^2_p = 0.37; n = 7-8 per group). To our surprise though, animals that received MK-801 did not demonstrate impaired fear memory, as could be expected if reconsolidation had been affected, but actually performed significantly better than controls.

Figure 2.3



*Percent freezing during test after different reactivation durations. MK-801 had no significant effect, except with 5 min condition. Data presented as mean + SEM. * p < 0.05 (MK-801×Control). n = 7-12/group.*

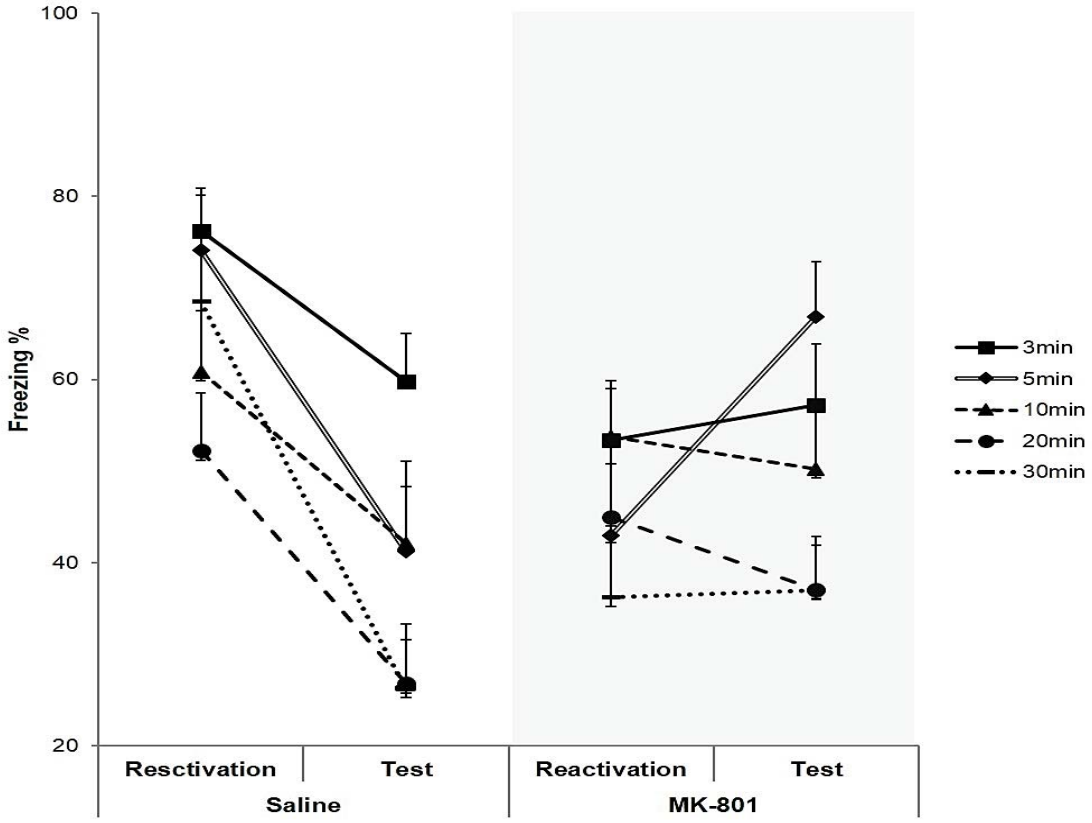
Thereafter, although memory was not sensitive to MK-801 during the intermediate conditions of reactivation, it could not be taken as evidence for the null-point since the parameters for reconsolidation and extinction were not evident with the protocol adopted here. Additionally, two-way ANOVA analysis of all factors together, revealed a main effect of drug ($F_{1,85} = 6.31$, $p = 0.014$; $\eta^2_p = 0.08$) and reactivation duration ($F_{4,85} = 7.68$, $p < 0.001$; $\eta^2_p = 0.29$), but no drug x duration interaction ($F_{4,85} = 1.29$, $p = 0.282$), further reinforcing that MK-801 did not have distinguishably effects over different reactivation durations as we have predicted.

These unexpected results though, rather than offer negative evidence for our hypothesis, could simply reflect a limited analysis of the freezing behaviour. In other words, evaluating memory expression only during test may have been insufficient to reveal the effects of MK-801 on memory. Therefore, we extended our analysis by examining the change on behaviour before and after animals were submitted to the different conditions of reactivation. That is, by comparing in each condition the expression of freezing during the start of reactivation (first 3 min) and test sessions (Fig. 2.4).

When analysing the control groups of the different reactivation conditions, in order to first establish the baseline between-session pattern of behaviour, repeated-measures ANOVA revealed a main effect of session ($F_{1,37} = 97.96$, $p = 0.000$; $\eta^2_p = 0.73$), reactivation duration ($F_{4,37} = 4.32$, $p = 0.006$; $\eta^2_p = 0.32$) and session x duration interaction ($F_{4,37} = 3.03$, $p = 0.030$; $\eta^2_p = 0.25$). Analyses of simple main effects elucidated an interesting, consistent decrease in freezing between reactivation and test with all reactivation conditions. Not only with the longer reactivation sessions (20 min: $F_{1,7} = 7.88$, $p = 0.026$; $\eta^2_p = 0.53$; $n = 8$) (30 min: $F_{1,6} = 44.61$, $p = 0.001$; $\eta^2_p = 0.88$; $n = 7$), where extinction and suppression of the fear memory would be expected, but also during the short and intermediate conditions of 3 min ($F_{1,11} = 14.82$, $p = 0.003$; $\eta^2_p =$

0.57; n = 12), 5 min ($F_{1,6}$ = 42.74, p = 0.001; η^2_p = 0.88; n = 7) and 10 min ($F_{1,7}$ = 13.34, p = 0.008; η^2_p = 0.66; n = 8). Although, it is important to mention that any interpretation here must be taken carefully, since freezing on reactivation may have been affected by the injection procedure that preceded this session.

Figure 2.4

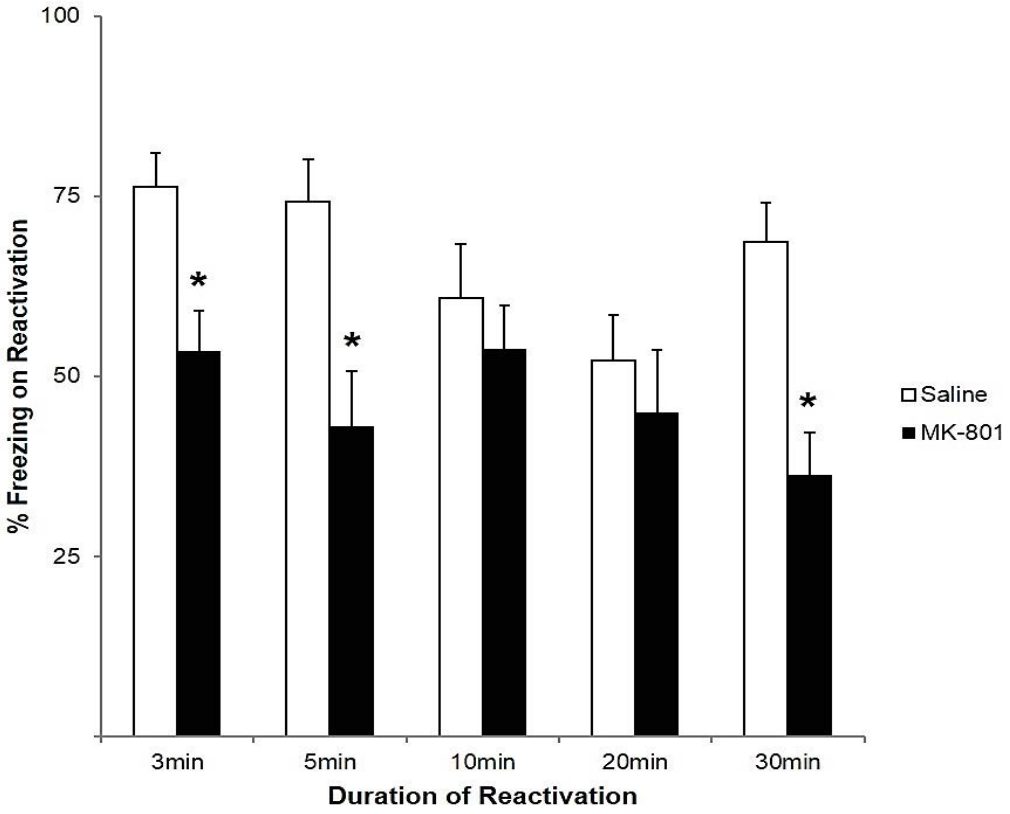


Freezing during reactivation (first 3 min), and test. There was significant difference in freezing between sessions in all conditions where animals received Saline ($p < 0.05$), but not when MK-801 was administered ($p > 0.05$), except with the 5-min condition ($p = 0.006$). Data is presented as mean + SEM. n= 7-12/group.

Nevertheless, when we analysed freezing across sessions of animals treated with MK-801, repeated-measures ANOVA revealed no main effect of session ($F_{1,39}$ = 1.26, p = 0.269) nor reactivation duration ($F_{4,39}$ = 2.32, p = 0.074), but a significant session x duration interaction ($F_{4,39}$ = 3.08, p = 0.027; η^2_p = 0.24), indicating differential effects of session within different reactivation durations. Simple main effect analysis confirmed a significant difference of freezing between reactivation and test when animals received MK-801 before a 5-min ($F_{1,7}$ = 14.90, p = 0.006; η^2_p = 0.68; n = 8), but no other reactivation durations ($F_{1,7\text{-}11,}$ p > 0.178; n = 8-12 per group). These results highlight two main observations: 1) that MK-801 treated animals did not express the same consistent decrease of freezing between reactivation and test observed in controls and, 2) that the administration of MK-801 before a 5-min reactivation not only prevented this decrease, but also led to significant higher freezing expression during test.

However, instead of indicating that MK-801 prevented any freezing decrease between sessions, those observations could actually represent an artefact from an already reduced freezing during reactivation, since MK-801 can acutely increase locomotor activity (Zemanova et al., 2013). Indeed, two-way ANOVA showed a main acute effect of drug during reactivation ($F_{1,86}$ = 23.97, p < 0.001; η^2_p = 0.24), and no effect of duration ($F_{4,86}$ = 2.08, p = 0.092), or drug x duration interaction ($F_{4,86}$ = 1.69, p = 0.162). Simple main effect analysis confirmed that animals treated with MK-801 expressed significantly lower freezing during the start of most reactivation conditions (3 min: $F_{1,23}$ = 9.80, p = 0.005; η^2_p = 0.31) (5 min: $F_{1,14}$ = 9.62, p = 0.008; η^2_p = 0.43) (30 min: $F_{1,14}$ = 15.70, p = 0.002; η^2_p = 0.55) as we can see in Fig. 2.5.

Figure 2.5



*Percent freezing during the start of the reactivation session (first 3 min). * p <0.05, saline x MK-801. Data is presented as mean + SEM. n= 7-12/group.*

Therefore, considering the acute effect of MK-801 on reactivation, and a possible interference of the procedure of injection that preceded the session, freezing expression during reactivation and any comparison with test do not seem to offer a reliable measurement of memory in this case where pharmacological manipulations preceded reactivation.

2.4 Discussion

In this study we have observed that MK-801 administered before contextual fear reactivation did not have significant effects upon memory with either short (3 min), long (30 min), or most of the intermediate (10 and 20 min) reactivation durations, except by the 5 min condition (Fig. 2.4). Additionally, there was a consistent decrease on memory expression between reactivation and test in the control groups of all experimental conditions, which was not observed when MK-801 was administered before the different reactivation sessions (Fig. 2.5). However, MK-801 was shown to also acutely impact freezing expression during most reactivation sessions (3, 5 and 30 min) (Fig. 2.6). Taken together, these results offer interesting insights, but do not support strong interpretations on what regards the *null-point* of memory, since the reactivation parameters for reconsolidation and extinction were not evident here.

In none of our conditions did pre-reactivation MK-801 result in a subsequent impairment of contextual freezing at test, which would be expected if reconsolidation had been affected. MK-801 and other NMDA antagonists have been reported to disrupt memory reconsolidation in the contextual fear conditioning (Brabant, Charlier and Tirelli, 2013; Ribeiro *et al.*, 2013; Lee and Flavell, 2014; Heath *et al.*, 2015) and several other learning paradigms (Brown *et al.*, 2008; Wu *et al.*, 2012; Alaghband and Marshall, 2013; Flint, Noble and Ulmen, 2013; Exton-McGuinness *et al.*, 2014; Vengeliene, Olevska and Spanagel, 2015). Here, however, we could not find consistent evidence for an amnesic effect of pre-reactivation MK-801 in the contextual fear memory.

It is possible that we did not find any effect of MK-801 on reconsolidation because the experimental parameters were not appropriate for this purpose, differently of the studies that did report an effect with similar, but not identical conditions. Brabant et. al. (2013) conducted the experiments in female mice, while here, male rats were used. Moreover, conditioning was much less intense (0.25mA), context re-exposure was shorter (2min), and MK-801 was administered post, and not pre-reactivation. Ribeiro et. al. (2013) used male rats, but from a different breed (Wistar), conditioning was also less intense (0.4mA), and drug was administered post-reactivation. Additionally, a different NMDA-antagonist was used (arcaine). The results reported by Lee and Flavell (2014) were observed with the same animal model used here. However, conditioning was also less intense (0.5mA), and MK-801 was injected post-reactivation. Besides, memory reactivation and destabilization were conducted under the effect of the CB1-agonist Arachidonyl-2-chloroethylamide (ACEA). Health et. al. (2015), used the same timing of drug administration used here (pre-reactivation) and same animal model. But, intensity of conditioning and reactivation duration were both different (0.5mA and 2min, respectively). On all these studies, where NMDA-antagonists were observed to impair reconsolidation of the contextual fear memory, we can notice that conditioning was less intense, and reactivation was shorter. Moreover, except by Health et al. (2015), administration of drug was conducted after reactivation, not before.

Considering that footshocks of higher potency during training is likely to result in stronger memories, and that stronger memories require longer or more intense reactivation in order to enter labilization/reconsolidation (Wang, de Oliveira Alvares and Nader, 2009; Winters, Tucci and DaCosta-Furtado, 2009; Alfei, Ferrer Monti, *et al.*, 2015), it is possible that the reactivation sessions used here were not long enough to trigger destabilization and bring memory to a sensitive state. This is unlikely though, because we did not find an amnesic effect of MK-801 only

with the short 3-min session, but also with the longer 5, 10, 20 and even 30-min conditions. Since there was no amnesic effect of MK-801 with context re-exposure of any duration, we may consider that the more intense training not only resulted in stronger memories, but also in an asymptotic learning which can be reasonably resistant to destabilization (Rodriguez-Ortiz *et al.*, 2005, 2008; Lee, 2010). We also do not think this is the case, considering the average freezing in the start of the reactivation sessions (first 3 min), although reasonably high, was in overall not near to maximal levels (57.74 ± 2.40), with only 2 in a total of 88 animals expressing more than 95% freezing. Moreover, many other studies with similarly intense and even stronger training, were able to induce memory destabilization with context re-exposure (Bustos, Maldonado and Molina, 2006; Abrari *et al.*, 2008; Taherian *et al.*, 2014)

Taking into account that most studies reporting reconsolidation impairment of NMDA-antagonists in the contextual fear conditioning made use of post-reactivation drug administration, it is possible that the different timing of injection used here contributed for the unexpected results. One reason for that could be the acute effect of MK-801 on freezing behaviour (Figure 2.5). Zemanova et al. (2013) observed before that MK-801 in similar doses (0.12 and 0.15 mg/kg) caused hyperactivity in Long-Evans rats. Considering that freezing involves the suppression of locomotion, it is reasonable to expect some degree of interference of MK-801 with the freezing behaviour during reactivation. The locomotor effect observed by Zemanova though, did not prevent efficient learning in an active place avoidance task when animals were familiar to the training arena. This may suggest that the reduced freezing we observed on animals administered with MK-801 could simply reflect an inability to express memory, due its effect on locomotor activity, rather than actual inhibition of memory reactivation and retrieval.

Nevertheless, there is evidence supporting that reexposure to conditioned stimulus can still induce memory reactivation, followed by destabilization and reconsolidation, even when memory is not actively expressed on behaviour. Rodriguez-Ortiz and colleagues in 2005, for example, observed that destabilization of a taste aversion learning does not particularly depend on the expression of the memory during reexposure to conditioned taste. Later, inhibition of AMPA receptors in the amygdala were shown to block the expression of conditioned freezing, but not the induction of memory destabilization (Mamou, Gamache and Nader, 2006; Milton et al., 2013). Therefore, although reactivation, destabilization, and expression of memory on behaviour are all linked processes, it appears to have a certain degree of independence among each other. That means the reduced freezing expression during context reexposure observed here with MK-801 does not necessarily imply memory destabilization was similarly affected.

Regarding NMDA receptors, antagonists as Ifenprodil and AP5 administered in the amygdala before reactivation were shown to prevent destabilization in the cued fear conditioning, without acutely affecting freezing behaviour (Mamou, Gamache and Nader, 2006; Hong et al., 2013). In addition, the subunits composition of the NMDAR in the amygdala has been observed to influence the ability of auditory fear to engage into destabilization. Conversely, pre-reactivation administration of the NMDA partial agonist D-cycloserine (DCS) was shown to facilitate destabilization of the contextual fear memory, without as well, affect memory retrieval nor expression (Bustos et al., 2010; Ortiz et al., 2014; Gazarini et al., 2015; Espejo et al., 2016). Therefore, NMDA receptors as in opposite to AMPA, appear to be involved in the engagement of memory labilization, but not reactivation or retrieval. These observations further reinforce the acute effect of MK-801 on freezing behaviour to be primarily a locomotion side-effect, rather than actual impairment on memory retrieval. On the other side, it also indicates MK-801 may still have had a concomitant effect on memory labilization. By stopping memory from

entering this process, pre-reactivation MK-801 may have paradoxically prevented its expected amnesic effect on reconsolidation, since memory was not susceptible to disruption.

Interestingly, despite the indications of NMDA involvement on memory destabilization, pre-reactivation MK-801 has been reported by Health et. al. (2015) to successfully impair reconsolidation in the contextual fear conditioning. The parameters used for training there were less intense though and may have resulted in a weaker memory, which as discussed before, are more prone to engage into labilization and reconsolidation. Therefore, even if MK-801 did have some effect on destabilization, it might not have been enough to prevent the process under these parameters. Moreover, it is important to notice this effect still requires to be replicated in further studies in order to rule out the possibility of a false positive result.

On what regards pavlovian memories, which involves the association of reward/punishment to discrete visual or auditory stimulus, there are several reports of reconsolidation impairment as a result of systemic pre-reactivation MK-801 (Lee, Milton and Everitt, 2006; Milton *et al.*, 2008; Flavell and Lee, 2013; Reichelt and Lee, 2013; Exton-McGuinness *et al.*, 2014; Exton-McGuinness and Lee, 2015). On the other side, NMDA antagonists administered locally in the amygdala has been observed to affect destabilization instead, when given before reactivation in pavlovian related paradigms (Hong *et al.*, 2013; Milton *et al.*, 2013; Reichelt, Exton-McGuinness and Lee, 2013). Additionally, manipulations of the NMDAr subunits composition in the amygdala has been described to significantly influence memory's likelihood of destabilization in the auditory fear conditioning (Holehonnur *et al.*, 2016). Therefore, considering localized inhibition of NMDA receptors prevent memory from entering destabilization, while systemic MK-801 does not, it is possible the absence of effect on these cases result from a poor action of the drug in the amygdala, when intra-peritoneally administered. This supposition,

however, is purely hypothetical and requires more experimental evidence in order to be fully considered.

A possible effect of pre-reactivation MK-801 on memory destabilization may also offer some interesting insights on the results observed with the 5-min condition. It has been shown before that in the presence of CS-US reinforcement, reactivation followed by destabilization and reconsolidation may lead to memory strengthening (Lee, 2008; Inda, Muravieva and Alberini, 2011; De Oliveira Alvares *et al.*, 2013). Additionally, there is evidence that timing of US-deliver during training influences the temporal expectation of US presentation during reactivation (Alfei, Monti, *et al.*, 2015). Thereafter, in an event where reinforcement is not present *for longer than expected*, yet not long enough to certainly represent a new CS-no-US learning, we could still expect some degree of weakening in the CS-US association intensity. Considering that during the 5-min reactivation session animals did not receive reinforcement for considerably longer than what could be expected, i.e. 3 min, it is possible that the reduced freezing of controls during test resulted from a weakened context-shock expectation, mediated by reconsolidation. Reconsolidation has been reported in other studies to result in memory attenuation when reactivation was coupled with certain events as, the presentation of positive appetitive stimulus (Haubrich *et al.*, 2015), parallel extinction training (Monfils *et al.*, 2009), and distractor tactile stimuli (Crestani *et al.*, 2015). Therefore, it is possible *weakening*, together with strengthening, addition of new information and precision keeping (Lee, 2008, 2010; De Oliveira Alvares *et al.*, 2013), is one more potential outcome of an overall updating purpose of the memory reconsolidation phenomenon.

The possibility that the 5-min reactivation here induced a reconsolidation-mediated weakening of the fear memory, and, the possibility that MK-801 administered before reactivation affected

the engagement of memory into destabilisation, may offer together a reasonable explanation for the results observed with the 5-min condition. On this scenario, animals treated with MK-801 would be expected to express greater freezing than controls during test because, in this case, memory would not have undergone the weakening process hypothetically dependent on memory labilization. It is important to mention though that the experiments here were not designed to study the role of NMDA receptors on memory destabilization, nor a weakening process that could supposedly be mediated by reconsolidation, meaning the results observed do not allow for more than speculative discussions on that matter.

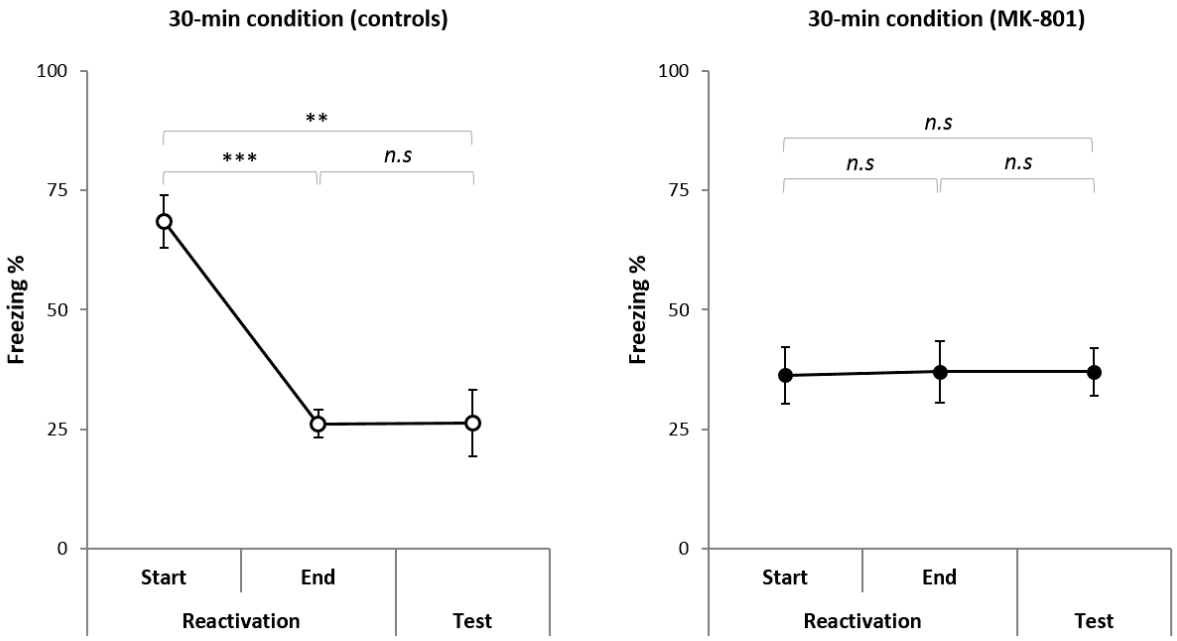
Nevertheless, these results offer interesting insights that could be further explored with appropriate experimentation. Administration of general and better-established destabilization-blockers as Nimodipine or Ifenprodil (Suzuki *et al.*, 2008; De Oliveira Alvares *et al.*, 2013; Crestani *et al.*, 2015; Haubrich *et al.*, 2015) before reactivation in which parameters favourite weakening of the CS-US association (as the 5-min condition studied here), in conjunction with respective no-reactivation control groups for baseline comparison, should provide stronger and more reliable evidence on whether memory weakening mediated by a destabilization/reconsolidation process is really a viable phenomenon. Conversely, in order to better understand the role of NMDA receptors in the labilization of contextual fear memories, administration of antagonists locally in the hippocampus and/or amygdala, in order to prevent possible non-mnemonic confusing effects of a systemic NMDA inhibition, associated with well-known pharmacological and behavioural manipulations that can lead to destabilization-dependent modifications on memory such as disruption, updating and strengthening (Debiec, LeDoux and Nader, 2002; Lee, 2008, 2010; Nader and Hardt, 2009), should provide a clearer understanding on the role of NMDA in the destabilization process.

On what regards the longer reactivation session of 30 min, we were expecting treatment with MK-801 to result in extinction impairment, which would be reflected during test as significantly higher freezing expression. However, no significant difference was observed between groups during the test session, which, could indicate that (1) the reactivation parameters were not sufficient to engage memory extinction; (2) reactivation was sufficient to engage extinction, but, extinction was not sensitive to pre-reactivation MK-801 treatment; or (3) extinction was engaged and sensitive to MK-801, but, its effects were not behaviourally and/or statistically evident in this study.

Considering we did not observe significant effect of MK-801 with the condition we expected to observe extinction, it is possible that for the parameters used here, 30-min context reexposure was still not long enough to induce effective extinction learning, or at least, not robustly. If there was no proper extinction learning, no effect of drug injection would actually be expected, since there would be no process to be affected. However, many studies using contextual fear conditioning of similar intensity (0.5-0.7mA footshocks) have reported long-term extinction, sensitive to pharmacological interventions, with reactivation sessions varying from 20 to 30 minutes (de Oliveira Alvares *et al.*, 2008; Fiorenza *et al.*, 2012; de Carvalho Myskiw *et al.*, 2015; Schmidt *et al.*, 2015; Haubrich *et al.*, 2017; Lunardi *et al.*, 2018). Moreover, when analysing freezing expression of the controls in the beginning and the end of the reactivation session, we can observe significant freezing decrease ($p = 0.001$), suggesting the reactivation procedure was capable of promoting some extinction learning (Fig. 2.6a). In addition, 24h later freezing remained decreased ($p = 1.000$; end-react x test), and, significantly lower than the first context reexposure ($p = 0.002$; test x start-react). Although the pattern of change on freezing behaviour alone does not offer strong evidence for a memory extinction process, this, in

conjunction with the literature and the other results presented here, suggests extinction at some extend was engaged in the 30-min reactivation condition.

Figure 2.6



Percent freezing acrooss start (first 3 min) and end (last 3 min) of 30-min reactivation and the test session, on animals treated previously with ***a)*** *saline (controls) and* ***b)*** *MK-801. n.s: non-significant. ** p <0.01. *** p <0.001. Data is presented as mean + SEM. n= 7-12/group.*

If extinction effectively occurred with the 30-min condition, then, the absence of significant difference between Saline and MK-801 groups might indicate the process was not sensitive to the adopted pharmacological intervention, either because the process was in overall independent of the NMDA receptor or, it was insensitive to the administration of MK-801 for different reasons as the timing of injection, via of drug administration and/or particular features of the contextual fear conditioning. Memory extinction has been shown in many studies to depend on NMDA receptors (Szapiro *et al.*, 2003; Gomes *et al.*, 2010; Fiorenza *et al.*, 2012; Corcoran,

Leaderbrand and Radulovic, 2013; de Carvalho Myskiw *et al.*, 2014), and therefore, it is unlikely MK-801 did not have an effect here because NMDA was not necessary during extinction. Accordingly, pre-reactivation systemic MK-801 has been reported before to impair extinction of spatial memories in the contextual fear conditioning (Song *et al.*, 2016) and conditioned place preference (Gass and Olive, 2010; Williams and Harding, 2014), in addition to several other reports involving aversive and appetitive pavlovian paradigms (Baker and Azorlosa, 1996; Lee, Milton and Everitt, 2006; Graham and Richardson, 2011; Flavell and Lee, 2013). Considering that, it does not seem that either the timing of injection (pre-reactivation), via of administration (systemic), or memory paradigm (contextual fear conditioning), represents particular issues in the experimental design used here.

However, it is worth noticing the effect reported for the contextual fear conditioning in Song. et. al. (2016) was observed with a less intense conditioning protocol (2 x 0.5 m.A footshocks), among other fundamental differences on the experimental design. More importantly, when stronger conditioning was used (6 x 0.5 mA footshocks), the same MK-801 injection had no significant effect upon memory extinction. Considering that the only difference between the two conditions was the strength of the conditioning, expressed as the number of footshocks received, it is possible that memory strength represents an important factor on how extinction responds to MK-801 treatments. It might be the case, for example, where more extensive extinction training, with longer or additional context reexposure sessions, and/or higher doses of the drug, would be required for the extinction of stronger memories to become effectively sensitive to MK-801.

It is also important to mention again the acute effects of MK-801 on the locomotor activity, that might represent a particular problem for the contextual fear conditioning. In the contextual fear

conditioning, memory expression depends on the engagement of freezing behaviour in response to a complex and sustained spatial stimulation, differently from aversive pavlovian paradigms for example, where we find more consistent reports on pre-reactivation MK-801 (Baker and Azorlosa, 1996; Lee, Milton and Everitt, 2006; Graham and Richardson, 2011). When we compare freezing expression of MK-801 treated animals with controls during the extinction training, we can observe freezing was significantly affected in the start of the session. Although by the end of the session MK-801 animals were expressing freezing at a similar level as controls ($p = 0.165$), the reduced freezing could still result from the locomotor effects of the MK-801 pre-treatment, and not from actual extinction learning. In other words, by affecting freezing behaviour during re-exposure to the conditioned context, pre-reactivation MK-801 might have also impaired extinction learning. Whether due pure locomotor effect of the drug, impaired within-session extinction, or, a combination of both factors, we do not observe on MK-801 animals the same extinction curve observed on controls (Fig. 2.6b). With extinction during context reexposure being somewhat affected, we could expect the subsequent effects of MK-801 to be attenuated and less evident, what could explain the absence of significant difference in the test session.

In addition, the parameters required to induce extinction of a relatively strong memory in the contextual fear conditioning (that is, relatively long duration of context reexposure), coupled with the pre-reactivation injection time (30 min before the beginning of the session) and the pharmacokinetics of MK-801, may have together affected the ability of MK-801 to impair memory extinction. MK-801 (0.1mg/kg) administered systemically in the rat has been shown to reach maximum concentration in the brain (14 nM) 40-60 min after injection, and to slowly decline bellow receptor affinity (3 nM) 120-140 min later (Wegener *et al.*, 2011). Considering that, we could expect MK-801 to still affect NMDA activity in the brain for at least one hour

after the end of the reexposure session, in the 30-min condition. This effect, though, would be in progressive decline, since the maximum concentration of the drug would have been reached just before the end, or by the end, of the session. Taken together, these observations indicate that in the 30-min condition, MK-801 effect upon extinction may have been partial and not powerful enough to be significantly reflected on behaviour.

Finally, despite all the potential issues discussed previously, it is still possible that extinction in the 30-min condition was engaged and disrupted by MK-801, but, the effect was not evident statistically for other technical reasons, as insufficient sample size and/or increased variability on data. We can observe a small tendency for a difference between groups in the test session, with MK-801 treated animals freezing on average 10% more than the control group. If we simulate a bigger sample size by triplicating the data and analysing it again (n = 21-24), this difference does become statistically significant (p = 0.025). If instead, we simulate a reduced variability on data by excluding potentially outlier samples, i.e., the animals with the lowest and the highest freezing score in each group, we can also observe a significant effect of MK-801 (p = 0.045) with a n as small as 5-6 per group. However, the effect remains mild and the difference between groups, not greater than 10%. Therefore, although the sample size and data variability may have prevented the observation of a significant effect of MK-801 in the 30-min condition, it does not seem to be the reason for the modest effect we observed on behaviour.

Regarding the intermediate reactivation sessions of 10 and 20 min, as in the other conditions, we did not find any significant effect of MK-801. Although with the intermediate conditions we were expecting to observe no effect of MK-801, accordingly to the null-point hypothesis, the results observed cannot clearly support, nor refute, the hypothesis. Since the parameters for either reconsolidation or extinction were not replicated in any of the conditions, it is not clear

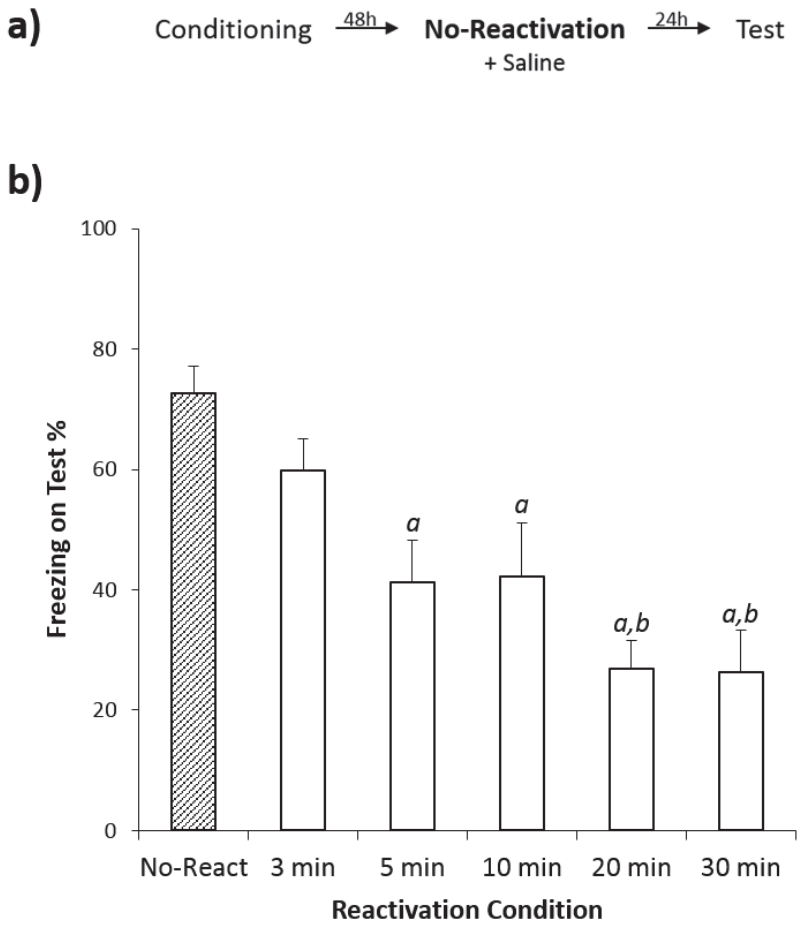
whether the absence of effect with the 10- and 20-min results from a transitional period from reconsolidation to extinction (i.e. the null-point), or, a failure to detect memory reconsolidation or extinction for any of the reasons mentioned in the 3- and 30-min conditions.

Despite the negative results observed, and all related implications and issues that comes with this kind of findings (Miller-Halegoua, 2017), some positive observations stands out from the collective of data presented here. One, a consistent decay of freezing after non-reinforced re-activation of any duration in animals treated only with saline (Fig. 2.4 – left panel), and two, an acute effect of MK-801 injection upon behaviour (Fig. 2.5).

Decrease on memory expression as a result of non-reinforced CS presentation is usually associated with extinction learning (Dunsmoor *et al.*, 2015a). Here, however, we observed that freezing between sessions declined even with a single context reexposure for as short as 3 and 5 min, which as discussed before, would unlikely trigger extinction. Considering that, and the presence of this effect in all conditions despite of the reactivation duration, it is possible that the decay in freezing here did not result from memory reactivation, but from other factors such as normal forgetting resulting from the simple passage of time from conditioning to test (3 days) (Hardt, Nader and Wang, 2013), which was a constant factor across all conditions. If the reduced freezing during the test session was a result of normal memory decay and not of non-reinforced memory reactivation, then, we would expect freezing to reach similar levels whether animals have been exposed to reactivation or not. To test that, we conducted additional data collection where animals were submitted to the exactly same experimental conditions used before (including saline/MK-801 injections), except by the reactivation session.

As we can see in Figure 2.7, freezing of non-reactivated animals was significantly higher than animals submitted the day before to 5-, 10-, 20- or 30-min reactivation sessions ($p \leq 0.013$). However, no difference was observed between non-reactivated controls and the 3-min condition ($p = 0.348$), indicating that in this case the decay on freezing between reactivation and test may have resulted from the passage of time, and not from the brief non-reinforced context reexposure.

Figure 2.7



[a]Significant difference in comparisson to the No-React condition ($p < 0.005$).
[b]Significant difference in comparisson to the 3-min condition ($p < 0.005$).

We may also have to consider the injection procedure that preceded the reactivation session and its potential effect on memory. Stress and related hormones have been reported before to acutely impair reconsolidation (Abrari *et al.*, 2008; Meir Drexler and Wolf, 2017) and enhance memory extinction (Sawamura *et al.*, 2016; Meir Drexler, Hamacher-Dang and Wolf, 2017), leading, in both situations, to subsequent reduced fear expression. If we consider the intraperitoneal injection as a potential stressor, there is a possibility the procedure *per se* had some impact on either reconsolidation or extinction, following the reactivation sessions, causing or contributing for the freezing decay we observe between sessions of all conditions (Fig. 2.4). If this was the case, then we would expect animals exposed to reactivation, but not i.p injections, to express less or no decay in freezing during a test session. On the other side, if the phenomenon was mainly or exclusively caused by the non-reinforced memory reactivations, we would still observe the same results on both injected and non-injected animals. Therefore, additional experimentation would still be required in order to clarify the mechanisms behind the phenomenon observed.

Nevertheless, although in all conditions we observe decline in freezing, the decline magnitude was not equal across groups and resulted in significantly different level of freezing in the test session ($F_{4,41} = 4.99$, $p = 0.003$). This, in addition to the particular features of each reactivation condition discussed previously, may additionally suggest the common effect on behaviour (i.e. reduced freezing) may have resulted from similar, but fundamentally different phenomena across the different reactivation conditions. For instance, normal memory decay related to the passage of time, memory weakening mediated by non-reinforced reconsolidation, and memory suppression as a result of extinction learning. However, it is important to notice some of the difference observed on test might also be attributed to pre-existent differences between groups, since freezing at the start of reactivation was already not completely homogenous across conditions ($F_{4,41} = 2.84$, $p = 0.038$). See Fig. 2.4.

In summary, despite offering interesting insights, the results observed here do not allow clear conclusions on that matter, especially on what regards the null-point of the contextual fear memory, the original main objective of this study. Since a great number of the issues discussed seemed to result from the administration of drug before reactivation, we decided to review our experimental design and change the timing of injections, which will be discussed in the following chapter. By using post-reactivation administration of MK-801, we should still be able to clearly address both memory reconsolidation and extinction, as well as the transition between these processes, without though, the potential disruptive influence of the pre-reactivation injections we observed in the contextual fear conditioning.

Chapter III

ON THE TRANSITON OF

RECONSOLIDATION TO EXTINCTION

Part B

3.1 Disclosure

This chapter comprises the manuscript published in the Learning & Memory journal, and its associated supplemental material, as follows: *Cassini LF, Flavell CR, Amaral OB, Lee JLC. On the transition from reconsolidation to extinction of contextual fear memories. Learn Mem. 2017 Aug. 16;24(9):392-399. doi: 10.1101/lm.045724.117.*

It will be presented in the publisher`s format for clarity, which contains collaborative work. I am the primary author though and have solely planned and designed the study, collected all the data and conducted its analysis. I have also written the first draft of the manuscript, which was then edited by the co-authors, including my supervisor Jonathan Lee. Moreover, we call attention for the data presented on Figure 2 of this chapter and the non-reactivation data mentioned on text, which are the same presented on the chapter II, not experiments replication. Nevertheless, these results are only complimentary and have not been nor will be published in duplicate.

The manuscript presented here can also be found at:

http://learnmem.cshlp.org/content/24/9/392

Supplemental material can be found here:

http://learnmem.cshlp.org/content/suppl/2017/08/09/24.9.392.DC1

Manuscript

On the transition from reconsolidation to extinction of contextual fear memories

Lindsey F. Cassini, Charlotte R. Flavell, Olavo B. Amaral, et al.

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Research

On the transition from reconsolidation to extinction of contextual fear memories

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Retrieval of an associative memory can lead to different phenomena. Brief reexposure sessions tend to trigger reconsolidation, whereas more extended ones trigger extinction. In appetitive and fear cued Pavlovian memories, an intermediate "null point" period has been observed where neither process seems to be engaged. Here we investigated whether this phenomenon extends to contextual fear memory. Adult rats were subjected to a contextual fear conditioning paradigm, reexposed to the context 2 d later for 3, 5, 10, 20, or 30 min, with immediate injections of MK-801 or saline following reexposure, and tested on the following day. We observed a significant effect of MK-801 with the 3- and 30-min sessions, impairing reconsolidation and extinction, respectively. However, it did not have significant effects with 5-, 10-, or 20-min sessions, even though freezing decreased from reexposure to test. Further analyses indicated that this is not likely to be due to a variable transition point at the population level. In conclusion, the results show that in contextual fear memories there is a genuine "null point" between the parameters that induce reconsolidation and extinction, as defined by the effects of MK-801, although NMDA receptor-independent decreases in freezing can still occur in these conditions.

[Supplemental material is available for this article.]

The retrieval of an associative memory can result in different outcomes. Retrieval in the absence of further reinforcement can destabilize a memory, requiring a process of reconsolidation (Nader and Hardt 2009), or can cause memory extinction through new inhibitory learning (Giustino and Maren 2015). The balance between destabilization and extinction appears to be influenced by the relative strength of learning and extent of nonreinforced retrieval (Eisenberg et al. 2003; Suzuki et al. 2004; Lee et al. 2006; de la Fuente et al. 2011; Flavell and Lee 2013). More extensive stimulus reexposure (i.e., extinction training), or weaker initial conditioning is more likely to result in extinction, whereas more restricted stimulus reexposure preferentially engages memory destabilization. This apparent competition between destabilization and extinction manifests as a bidirectional effect of amnestic treatment, depending of the parameters of conditioning and retrieval. Either reconsolidation is impaired to reduce subsequent memory expression, or extinction is disrupted to maintain expression of the original memory (Eisenberg et al. 2003; Suzuki et al. 2004; Lee et al. 2006; de la Fuente et al. 2011; Flavell and Lee 2013).

In both appetitive Pavlovian and conditioned fear memories, recent evidence has indicated that extinction per se does not prevent memory destabilization and reconsolidation. In cue–sucrose, cue–fear, and context–fear settings, there appears to be a reexposure period between the parameters that engage destabilization and extinction, in which there is no behavioral effect of amnestic treatment (Flavell and Lee 2013; Merlo et al. 2014; Alfei et al. 2015). This "limbo" or "null point" suggests that extinction itself is not a boundary condition on reconsolidation. However, the interpretation of the negative effect at the null point is not straightforward. While it has been argued that only a three-phase transition model with a null point can explain the behavioral data (Merlo et al. 2014), this assumes that the parameters of transition are the same across individuals, thereby implying that the absence of a drug effect reflects a genuine null point at an individual level. However, it is also possible that, while at the individual level there is a gradual or step function transition from destabilization to extinction, there are individual differences in the parameters of that transition, resulting in a lack of group effect at intermediate points. Namely, at these intermediate reexposure conditions, some individuals could be undergoing a destabilization/reconsolidation process, while others would have transitioned into extinction learning. This might be expected to manifest as a greater variability in behavior due to the existence of different subgroups at the null point; however, this is unlikely to be identified with the sample sizes that have been previously used. In the current study, we used larger cohorts of rats and used multiple analytical approaches in order to confirm the existence of the null point effect for contextual fear memories (Alfei et al. 2015) and disambiguate its potential explanations, namely: (1) that the null point is a genuine effect and represents a phenomenon at the individual level or (2) that the null point is an artifact of variation in the transition point between reconsolidation and extinction at the population level.

Results

CFC memory is insensitive to MK-801 between the parameters that induce reconsolidation and extinction

In order to confirm the existence of the null point in the reactivation of contextual fear memories, Lister-Hooded rats were subjected to a contextual fear conditioning (CFC) paradigm, consisting of training on day 1, context reexposure on day 3 and test on day

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4. The duration of the reexposure session varied across experiments, lasting for 3, 5, 10, 20, or 30 min. Immediately after reexposure, the NMDA receptor antagonist MK-801 was injected intraperitoneally (0.1 mg/kg). MK-801 is a well-known amnestic agent shown to have bidirectional outcomes upon behavior when affecting reconsolidation or extinction (Lee et al. 2006; Flavell and Lee 2013; Merlo et al. 2014). The aversive response (freezing) was automatically recorded during all sessions and used as an index of fear memory.

We found that in the short 3-min reexposure condition, while there was no difference between the experimental groups at the reexposure session itself (Fig. 1A; $F_{(1,26)} = 2.46$, $P = 0.129$; $n_p^2 = 0.09$; $BF_{10} = 0.87$), the MK-801 group showed significantly less freezing than the controls at test (Fig. 1B; $F_{(1,26)} = 6.96$, $P = 0.014$; $n_p^2 = 0.21$; $BF_{10} = 4.00$). This indicates that this short, nonreinforced context reexposure was sufficient to engage the destabilization of the previously conditioned contextual fear memory. The memory in turn, became sensitive to disruption by MK-801, resulting in impaired memory expression in the test session.

On the other hand, the administration of MK-801 after a reexposure session that lasted 10 times longer (30 min) resulted in significantly higher freezing in the test session when treated animals were compared with the control group (Fig. 1B; $F_{(1,40)} = 4.23$, $P = 0.046$; $n_p^2 = 0.10$; $BF_{10} = 1.58$). Again, there were no preexisting group differences at the beginning ($F_{(1,40)} = 0.43$, $P = 0.517$; $n_p^2 = 0.01$; $BF_{10} = 0.36$) or the end ($F_{(1,40)} = 0.23$, $P = 0.634$; $n_p^2 = 0.01$; $BF_{10} = 0.33$) of the 30-min reexposure session (Fig. 1A). These results suggest that MK-801 impaired the extinction of contextual fear memory, although the effect is rather weak (albeit statistically significant). This interpretation is consistent with the observation that context reexposure for 30 min was sufficient for a memory extinction process to take place and suppress the original CFC memory.

Interestingly, Figure 1B shows that MK-801 had no observable effect upon test freezing when administered after the intermediate context reexposures of 5 min ($F_{(1,18)} = 0.99$, $P = 0.333$; $n_p^2 = 0.05$; $BF_{10} = 0.57$), 10 min ($F_{(1,14)} = 0.32$, $P = 0.579$; $n_p^2 = 0.02$; $BF_{10} = 0.48$) and 20 min ($F_{(1,14)} = 0.79$, $P = 0.389$; $n_p^2 = 0.05$; $BF_{10} = 0.56$). Moreover, groups did not differ during the reexposure sessions in any condition, either at the beginning (5-min: $F_{(1,18)} = 0.75$, $P = 0.398$; $n_p^2 = 0.04$; $BF_{10} = 0.53$) (10-min: $F_{(1,12)} = 0.46$, $P = 0.511$; $n_p^2 = 0.04$; $BF_{10} = 0.52$) (20-min: $F_{(1,14)} = 0.64$, $P = 0.437$; $n_p^2 = 0.04$; $BF_{10} = 0.53$) or at the end (5-min: $F_{(1,18)} = 0003$, $P = 0.957$; $n_p^2 = 0.00$; $BF_{10} = 0.41$) (10-min: $F_{(1,12)} = 0.38$, $P = 0.549$; $n_p^2 = 0.03$; $BF_{10} = 0.51$) (20-min: $F_{(1,14)} = 0.61$, $P = 0.446$; $n_p^2 = 0.04$; $BF_{10} = 0.53$) of the sessions. This lack of MK-801 effect between the parameters that induced reconsolidation and extinction suggests the existence of a "null point" or "limbo" phenomenon for contextual fear memories, as shown previously in other tasks with the same drug (Flavell and Lee 2013; Merlo et al. 2014) or in CFC with a GABA-A agonist (Alfei et al. 2015).

Furthermore, by analyzing all the factors together with a two-way ANOVA, we observed a significant interaction between drug and duration of context reexposure ($F_{(4,112)} = 2.63$, $P = 0.038$; $n_p^2 = 0.09$; $BF_{10} = 1.55$), with a main effect of duration ($F_{(4,112)} = 2.56$, $P = 0.042$; $n_p^2 = 0.08$; $BF_{10} = 1.77$), but no effect of drug ($F_{(1,112)} = 2.40$, $P = 0.124$; $n_p^2 = 0.02$; $BF_{10} = 0.38$). This further strengthens the conclusion that the effect of MK-801 was dependent upon reactivation duration.

The CFC null point is not a result of late drug administration

While the use of post-re-exposure drug administration shows the effects to be specific to reconsolidation or to the consolidation of extinction learning, it does present a potential interpretative problem. Although MK-801 had effects in both the 3- and 30-min conditions, there remained the possibility that with the intermediate exposure duration, reconsolidation was engaged, but a post-session administration of MK-801 was too late to affect the reconsolidation process (Lee and Everitt 2008). In other words, the absence of effect of MK-801 with the 10-min session might have

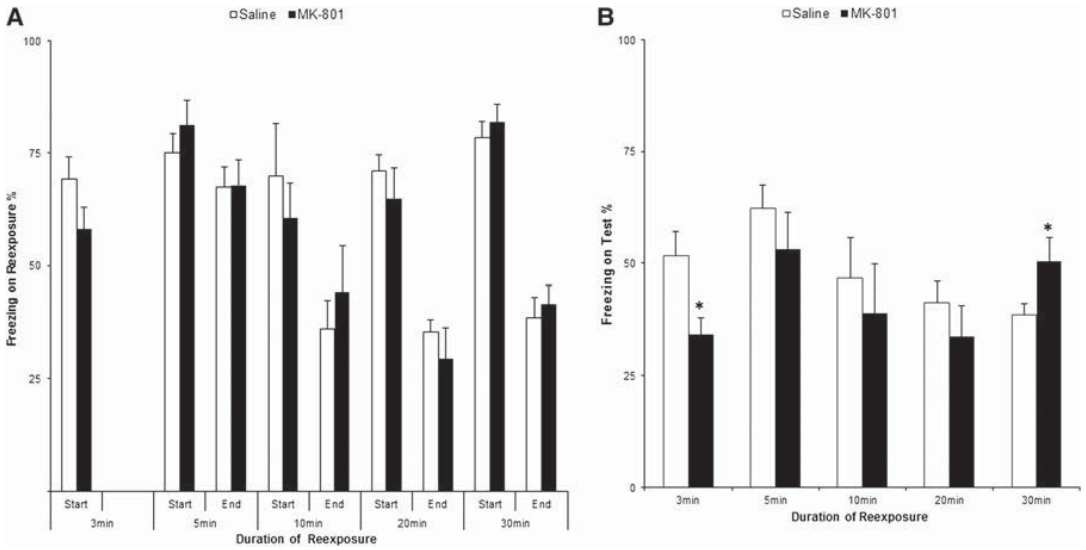

Figure 1. CFC memory is insensitive to MK-801 between the parameters that induce reconsolidation and extinction. Animals were subjected to Contextual Fear Conditioning and 2 d later, to a (*A*) context reexposure session of 3, 5, 10, 20, or 30-min. Immediately after, they received i.p. MK-801 or saline. Memory was assessed on the following day in a (*B*) test session. MK-801 had a significant effect when administered after 3 or 30 min, but no effect upon the intermediate conditions of 5, 10, and 20 min. There were no preexisting differences between groups during the start (first 3 min) or the end (last 3 min, or last 2 min in the 5-min condition) of the reexposure sessions in any condition. Data presented as mean + SEM. (*) $P <$ 0.05 (MK-801 × Control). $n = 14$ (Sal-MK/3 min), 13 (Sal/5 min), 7 (MK/5 min), 8 (Sal-MK/10–20 min), 20 (Sal/30 min), and 22 (MK/30 min).

been due to the fact that it would be too late to stop a process of memory reconsolidation that would have already happened by the time the drug became systemically available. To investigate if this could be the case, we used a 30-min prereactivation injection of MK-801, which has been demonstrated to impair reconsolidation across a number of paradigms (Lee et al. 2006; Brown et al. 2008; Flavell and Lee 2013), prior to the 10-min condition. Therefore, if reconsolidation were engaged by the 10-min context reexposure, a presession injection of MK-801 would be expected to impair reconsolidation to result in subsequent amnesia.

Animals that received MK-801 froze at equivalent levels at test as those treated with saline (Fig. 2; $F_{(1,14)} = 0.53$, $P = 0.478$; $n_p^2 = 0.04$; $BF_{10} = 0.51$). There was no difference either at the beginning of context reexposure ($F_{(1,14)} = 0.54$, $P = 0.473$; $n_p^2 = 0.04$; $BF_{10} = 0.51$) or at the end ($F_{(1,14)} = 0.06$, $P = 0.804$; $n_p^2 = 0.01$; $BF_{10} = 0.44$). Together with the previous data (Fig. 1), it is apparent that the CFC memory was insensitive to NMDA receptor antagonism irrespective of whether MK-801 was administered prior to or immediately after a 10-min context reexposure session. Therefore, there appears to be a genuine lack of amnestic effect of MK-801 with a reexposure session of this duration.

The CFC null point does not result from individual/subgroup differences

In order to determine whether the lack of group effect of MK-801 at the intermediate 10-min condition reflects individual differences in the transition from reconsolidation to extinction, we replicated the experiment with large cohorts of rats ($n = 19$–21 per group). Our primary approach to address population effects was to focus on the correlation between freezing levels in the test and reexposure sessions. We would expect both parameters to correlate in controls, as animals with low freezing at the end of the reexposure session would be expected to freeze less in the test session as well. In contrast, if MK-801 impairs between-session extinction or reconsolidation in specific animals, we would expect an alteration of that correlation. In this case, extinction blockade would likely lead to high test freezing in animals undergoing extinction (and thus presenting lower freezing) during the reexposure session, while animals undergoing reconsolidation (with presumably high freezing during context reexposure) would be expected to freeze less at the test. Again, MK-801 did not have any effect when analyzing the population as a whole ($F_{(1,38)} = 0.85$, $P = 0.362$; $n_p^2 = 0.02$; $BF_{10} = 0.43$) (Supplemental Fig. S2a). When we plot the freezing levels of animals at the end of context reexposure (as an index of within-session extinction) and test (as an index of between-session extinction), we indeed observe a positive correlation between sessions for the control animals (Fig. 3A; $r = 0.683$, $P = 0.001$, $BF_{10} = 35.14$). This correlation was not disrupted by the administration of MK-801 ($r = 0.534$, $P = 0.013$, $BF_{10} = 4.99$), and the slopes ($F_{(1,36)} = 0.09$, $P = 0.768$) and intercepts ($F_{(1,37)} = 2.51$, $P = 0.121$) of the two linear regressions were not statistically different.

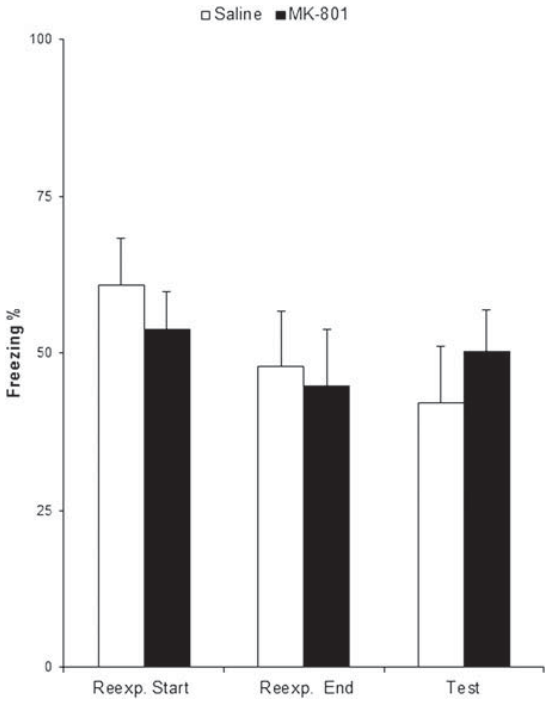

Figure 2. The CFC "null point" is not a result of late drug administration. Animals were subjected to contextual fear conditioning. Two days later, they received i.p. MK-801, or Saline, 30 min prior a reexposure session of 10 min. Memory was assessed on the following day in a test session. MK-801 had no significant effect. Data presented as mean + SEM. $n = 8$ per group.

Additional analyses compared freezing at test to freezing at the start of context reexposure (Fig. 3B), showing a positive, but nonsignificant correlation for both control ($r = 0.381$, $P = 0.107$, $BF_{10} = 0.95$) and MK-801-treated ($r = 0.444$, $P = 0.444$, $BF_{10} = 1.83$) groups. A comparison of the slopes of these nonsignificant correlations revealed no difference in their slopes ($F_{(1,36)} = 0.16$, $P = 0.692$) and intercepts ($F_{(1,37)} = 2.61$, $P = 0.115$). Given that freezing at the start of context reexposure is variable across rats, it is possible that an index of the decline in freezing over the course of the session is a more reliable measure of within-session extinction, and therefore we correlated such an index with test freezing (Fig. 3C). Surprisingly, there was no significant correlation, either for animals that received saline ($r = -0.230$, $P = 0.344$, $BF_{10} = 0.43$) or MK-801($r = -0.044$, $P = 0.848$, $BF_{10} = 0.27$). For completeness, we compared the slopes and intercepts of these nonsignificant linear regressions, which revealed no difference in their slopes ($F_{(1,36)} = 0.25$, $P = 0.616$) and intercepts ($F_{(1,37)} = 0.61$, $P = 0.439$). Finally we correlated test freezing with performance in the elevated plus maze task, performed 1 wk before fear conditioning, in order to test whether baseline anxiety levels affected the impact of MK-801 (Supplemental Fig. S1). Again, no correlation was observed in either of the groups (Sal: $r = 0.206$, $P = 0.398$, $BF_{10} = 0.40$; MK-801: $r = 0.057$, $P = 0.806$, $BF_{10} = 0.28$) and slopes and intercepts did not differ ($F_{(1,36)} = 0.17$, $P = 0.682$ and $F_{(1,37)} = 0.81$, $P = 0.373$, respectively).

In order to confirm the findings of our analyses for the 10-min condition, we replicated them on a large cohort tested with the 5-min reexposure that also appears to fall within the null point (but without the baseline elevated plus maze). Once more, MK-801 did not have any effect when analyzing the population as a whole ($F_{(1,36)} = 0.35$, $P = 0.558$; $n_p^2 = 0.01$; $BF_{10} = 0.36$) (Supplemental Fig. S3a). Moreover, we observed a pattern of results similar to that observed with the 10-min analyses (Fig. 3D–F), indicating that there were no subpopulations of reconsolidating and extinguishing rats. Freezing at the end of context reexposure and test correlated positively for the control (Fig. 3D: $r = 0.457$, $P = 0.049$, $BF_{10} = 1.72$) and MK-801 animals ($r = 0.683$, $P = 0.001$, $BF_{10} = 35.07$), with the two linear regressions not differing in comparison of their slopes ($F_{(1,34)} = 0.92$, $P = 0.344$) and intercepts ($F_{(1,35)} = 0.17$, $P = 0.681$). Positive correlations were also observed between freezing at the start of reexposure and in the test session, and were more robust than those seen with 10-min condition (Sal: $r = 0.682$, $P = 0.001$, $BF_{10} = 34.45$; MK-801: $r = 0.530$, $P = 0.020$, $BF_{10} = 3.58$). Nevertheless, there was no significant difference in slope ($F_{(1,34)} = 0.04$, $P = 0.850$) or intercept ($F_{(1,35)} = 1.09$, $P =$

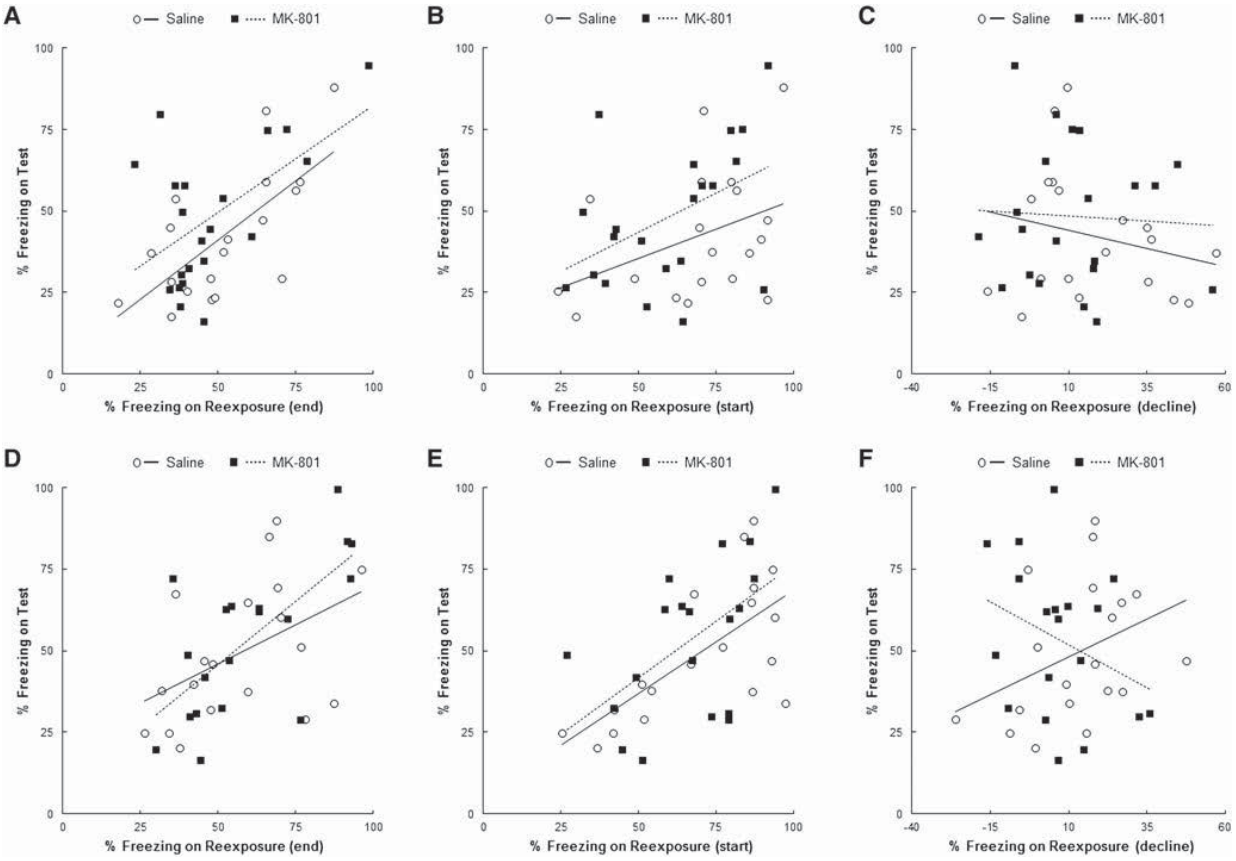

Figure 3. The CFC "null point" does not result from individual differences. Animals were subjected to contextual fear conditioning in big cohorts and 2 d later, to an intermediate reexposure session of 10 or 5 min. Immediately after, they received i.p. MK-801 or Saline and on the following day, memory was assessed in a test session. Freezing percentages during the test session were then correlated to (*A*) freezing at the end (last 3 min) of the 10-min reexposure session, (*B*) freezing at the start (first 3 min) of the 10-min reexposure session, (*C*) decline of freezing during the 10-min reexposure session (start–end), (*D*) freezing at the end (last 2 min) of the 5-min reexposure session, (*E*) freezing at the start (first 3 min) of the 5-min reexposure session, (*F*) decline of freezing during the 5-min reexposure session (start–end). *A*, *D*, and *E* show significant correlations between parameters for both Sal and MK-801 groups ($P < 0.05$). MK-801 did not significantly affect the slopes or the intercepts of any significant regressions. Data presented as mean + SEM. $n = 19$–21 per group.

0.302) of the two linear regressions. Finally, there was again no significant correlation between freezing decline across the brief reexposure session and freezing at test in either of the groups (Sal: $r = 0.379$, $P = 0.110$, $BF_{10} = 0.93$; MK-801: $r = -0.326$, $P = 0.173$, $BF_{10} = 0.67$). While the statistical comparison between slopes revealed a significant difference ($F_{(1,34)} = 4.70$, $P = 0.037$; the magnitude of the difference does not allow the comparison of intercepts), this is likely a chance finding, as both correlations are weak and nonsignificant, as observed in the 10-min condition.

As an alternative analytical approach, we stratified the large cohorts of rats, for both the 10- and 5-min conditions, into subgroups, based upon baseline anxiety (high versus low), freezing at the start of the reexposure session (high versus low), freezing at the end of reexposure (high versus low), freezing decline across the reexposure session (small versus large) and freezing during the test itself (high versus low). Thereafter, CFC memory and the effect of MK-801 on the test session were analyzed across the different subpopulations of animals. None of these subpopulation analyses suggested the existence of divergent effects of MK-801 in different individuals (Supplemental Figs S2, S3).

Finally, we compared the variability in test freezing between the MK-801 and saline control groups for both the 10- and 5-min larger cohort experiments. Levene's test revealed that there was no difference between the groups' variances, either in the 10-min ($F_{(1,38)} = 0.34$, $P = 0.562$) or in the 5-min condition ($F_{(1,36)} = 0.26$, $P = 0.610$). The fact that the test variances between the saline and MK-801 groups are similar offers further evidence against the idea that MK-801 could be affecting reconsolidation in some animals and extinction in others during the null-point.

While there was no evidence for subpopulation differences in susceptibility to reconsolidation or extinction at intermediate reexposure durations, the analyses revealed an interesting pattern of consistent reductions in freezing from context reexposure to test, regardless of the duration of the reexposure. When comparing freezing between the start of reexposure and the test session in control rats from the first experiment (Figs. 1, 4), a repeated-measures ANOVA revealed a main effect of session ($F_{(1,57)} = 87.24$, $P < 0.001$, $n_p^2 = 0.61$; $BF_{10} = 3.97$), no effect of reexposure duration ($F_{(4,57)} = 1.37$, $P = 0.256$, $n_p^2 = 0.09$; $BF_{10} = 0.17$) and a significant duration × session interaction ($F_{(4,57)} = 4.94$, $P = 0.002$, $n_p^2 = 0.26$; $BF_{10} = 6.51$). Analyses of simple main effects confirmed a reduction in freezing with the longer 10-, 20-, and 30-min conditions ($P < 0.05$, $n_p^2 > 0.60$; $BF_{10} > 2.00$). While there was only a marginal effect of reduced freezing with the 5-min reexposure ($P = 0.080$, $n_p^2 = 0.23$; $BF_{10} = 1.89$), there was a significant reduction after the 3-min condition ($P < 0.001$, $n_p^2 = 0.75$; $BF_{10} = 368.59$), indicating

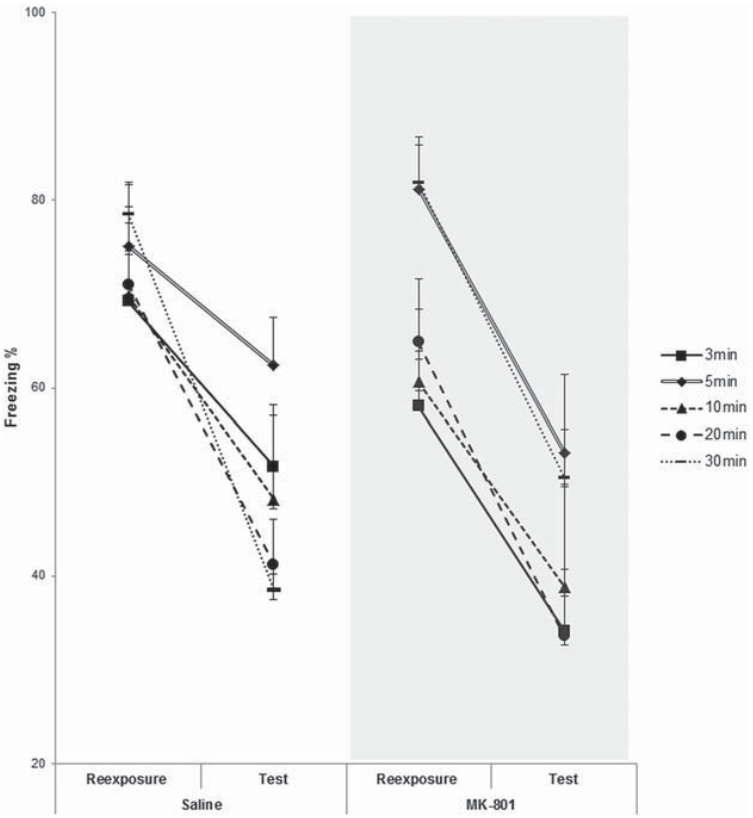

Figure 4. Consistent reductions in freezing from reexposure to test. Analysis of the freezing at the start of context reexposure (first 3-min) and at test from the experiment in Figure 1. There were consistent reductions in freezing in all conditions (except for Sal 5-min). Data is presented as mean + SEM. n = 14 (Sal-MK/3 min), 13 (Sal/5 min), 7 (MK/5 min), 8 (Sal-MK/10–20 min), 20 (Sal/30 min), and 22 (MK/30 min).

that context reexposure without reinforcement can result in some degree of decrease in freezing from reexposure to test even with short durations. By performing the same comparisons in the MK-801 animals, we observe a main effect of session ($F_{(1,54)}$ = 82.06, $P < 0.010$, $n_p^2 = 0.59$; $BF_{10} = 1.31$), an effect of reactivation duration ($F_{(4,54)} = 4.15$, $P = 0.005$, $n_p^2 = 0.24$; $BF_{10} = 4.83$) and no significant duration × session interaction ($F_{(4,54)} = 0.50$, $P = 0.738$, $n_p^2 = 0.01$; $BF_{10} = 2.57$). Analyses of simple main effects confirmed a reduction in freezing from reactivation to test with all reactivation durations ($P < 0.010$, $n_p^2 > 0.55$; $BF_{10} > 5.00$). It is notable that even with the 30-min condition, in which MK-801 animals froze significantly more than the controls (Fig. 1B), a decrease in memory expression from reactivation to test was still observed. Moreover, the freezing behavior in nonreactivation control groups (Sal = 74 ± 5, n = 19) (MK-801 = 71 ± 5, n = 17) confirmed that reactivation of any duration resulted in decreased freezing at test, in spite of MK-801 treatment.

Discussion

In this study, we have demonstrated that MK-801 impaired contextual fear memory reconsolidation with a short reexposure duration, and disrupted extinction with a long reexposure duration, as shown previously with other drugs in contextual fear conditioning (Suzuki et al. 2004; Bustos et al. 2009; Alfei et al. 2015) and with the same drug in other paradigms (Lee et al. 2006; Merlo et al. 2014). At intermediate durations of context reexposure, MK-801 had no observable effect on the expression of the contextual fear memory. This lack of effect was not due to the timing of MK-801 administration, as it was replicated with presession injection of the drug. Moreover, there was no evidence for subpopulations of animals responding differently to MK-801 at the intermediate reexposure duration. These results suggest that there is a period during the transition from reconsolidation to extinction where memory is indeed not sensitive to disruption.

The null point between reconsolidation and extinction has previously been demonstrated for appetitive Pavlovian memories (Flavell and Lee 2013) and cued fear memories (Merlo et al. 2014), as well as for the contextual fear memories studied here (Alfei et al. 2015). In each of these studies, one intermediate parameter of memory reactivation was found, in which amnestic treatment did not either impair or enhance subsequent memory expression at test. While two of the previous studies used the same dose of MK-801 as used here (Flavell and Lee 2013; Merlo et al. 2014), the third used systemic injections of midazolam (Alfei et al. 2015). Therefore, the existence of the null point is not unique to the use of MK-801 or to NMDA receptor antagonists.

In the current study, there was evidence for an extended null point period between the reexposure durations that induce reconsolidation and extinction. Context reexposures of 5, 10, and 20 min each revealed a lack of effect of MK-801. This extended duration in itself suggests that the null point cannot be explained simply by variability in the point of transition between reconsolidation and extinction across different animals, as one would expect at least some trend toward reconsolidation impairment at the 5-min end and extinction impairment at the 20-min end. Moreover, we predicted that the existence of subgroups showing impaired reconsolidation or extinction would manifest as (a) a reduction in the correlation between freezing levels in the reexposure and test sessions in treated animals, (b) an effect of MK-801 when analyzing subpopulations determined by one or more factors (e.g., levels of freezing at context reexposure or test, or extent of within-session extinction during reexposure), or (c) increased variability in the MK-801-treated rats compared with saline-treated controls. None of these predictions were supported by our data. Therefore, we conclude that the null point represents a period at which MK-801 impairs neither reconsolidation nor extinction (Fig. 5), in accordance to the three-phase transition model outlined by Merlo et al. (2014).

Contextual fear memory reconsolidation and extinction have both been demonstrated to be critically dependent upon NMDA receptor activity (Suzuki et al. 2004; Lee and Hynds 2013; Lee and Flavell 2014). The bidirectional effect of the same amnestic treatment, dependent upon the parameters of memory reactivation, indicates that the dissociable effects are mediated by impairments in different mnemonic processes (Lee et al. 2006; de la

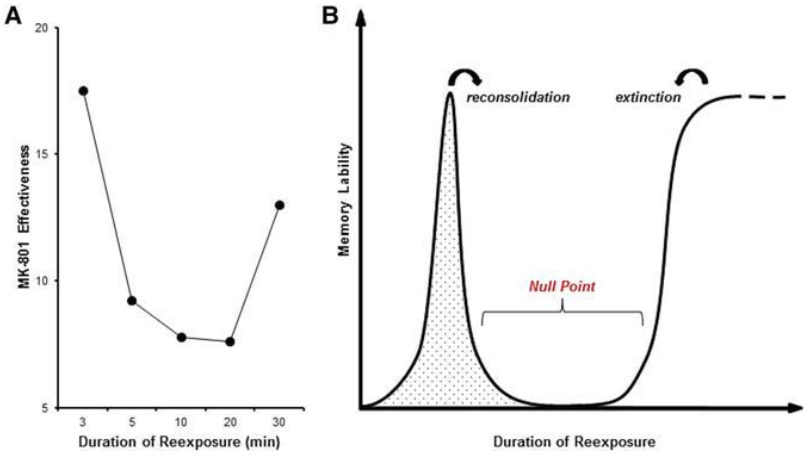

Figure 5. Three phase transition from reconsolidation to extinction. (*A*) Effectiveness of amnesic treatment (absolute difference in freezing between MK-801 and saline groups during test) across different conditions reveals a (*B*) three-phase model for the transition of reconsolidation to extinction of associative memories.

Fuente et al. 2011). This has led to the suggestion that there is a trace dominance effect, with the trace that is dominantly activated by memory retrieval being the one impaired by amnestic treatment, such as NMDA receptor antagonism or protein synthesis inhibition (Eisenberg et al. 2003). Mechanisms for such trace dominance have been postulated by computational models, in which different degrees of similarity between training and reexposure lead to reconsolidation or extinction-like phenomena (Osan et al. 2011; Gershman et al. 2017). However, these models do not predict a null point in which neither reconsolidation nor extinction is dominantly activated, leading to the lack of effect of MK-801. Our data, on the contrary, indicate that under conditions of no dominant trace activation, there is no disruptive effect of MK-801 on either reconsolidation or extinction in individual animals. Therefore, the start of NMDA receptor-dependent extinction per se does not seem to be the factor preventing memory reconsolidation. Instead, it appears that there could be independent mechanisms that suppress the engagement of reconsolidation, but are not by themselves sufficient to engage extinction. This may well be mediated at the cellular level (de la Fuente et al. 2011; Merlo et al. 2014), although we cannot rule out the possibility that the complex interplay between reconsolidation and extinction is regulated at the systems level, especially given that reconsolidation and extinction have only partially overlapping neural substrates (Bahar et al. 2004).

The current results also reveal a dissociation between the definitions of extinction as new learning vulnerable to amnestic treatment (e.g., MK-801) and as a long-term reduction of memory expression after reexposure (Pavlov 1927). It was notable that all reexposure durations resulted in a decline in contextual fear memory expression in the test session (Fig. 4). This was not due simply to the passage of time, as nonreexposed controls froze at higher levels than those undergoing reexposure. Therefore, while context reexposure led to behaviorally defined extinction for all durations, this extinction was apparently NMDA receptor-dependent only for the 30-min condition. Moreover, the reconsolidation impairment with the brief reexposure duration was observed in spite of a significant between-session decline in freezing, as has been previously documented in the literature (Charlier and Tirelli 2011; Brabant et al. 2013; Heath et al. 2015). Importantly, we observed a similar pattern of freezing reduction even in the animals that received MK-801 after context reexposure, no matter how long the session lasted for (Fig. 4). This extends even to the 30-min duration, indicating the presence of an NMDA receptor-independent process that weakens memory expression in these conditions. Indeed, the data from Merlo et al. (2014) show the same pattern of between-session memory decline in cued fear that was unaffected by MK-801 at their intermediate null point parameter. The fact that some degree of behaviorally defined extinction occurs in the absence of NMDA activity raises the question of what causes this freezing decline. Although it could be related to non-NMDA receptor-dependent extinction learning, which has been described in some conditions (Santini et al. 2001; Langton and Richardson 2008; Kim and Richardson 2010), or to delayed consolidation of extinction (i.e., beyond systemic availability of MK-801), it might also imply that behavioral extinction, at least in some cases, might involve not only learning of a new association, but also weakening of the original one (Barad 2006; Riebe et al. 2012; Almeida-Corrêa and Amaral 2014) through a process that might be less dependent on NMDA receptors than new learning.

Regardless of the uncertainty about the potential multiple mechanisms of weakening memory expression with extinction training, these observations and our wider results raise an important point about the transition between reconsolidation, the null point, and extinction. We could detect no reliable basis, other than systematically varying duration of context reexposure, upon which to predict whether a given duration will engage reconsolidation, NMDA receptor-dependent extinction or will fall into the null point. Certainly, there is no obvious pattern or threshold of memory decline that can distinguish between the parameters leading to reconsolidation and extinction. Previous studies of contextual fear memory showed that the parameters of the three-phase transition were partly dependent upon the timing of shock delivery during conditioning (Alfei et al. 2015), and that the parameters of reconsolidation depend upon memory age and strength (Suzuki et al. 2004). Although this was not tested directly, it is reasonable to predict that if older and stronger memories require more extended context reexposure to induce reconsolidation (Suzuki et al. 2004), the parameters of the null point and extinction will be similarly shifted to longer durations.

Previous studies have suggested a critical role for prediction error in triggering reconsolidation across a number of paradigms (Reichelt and Lee 2012; Díaz-Mataix et al. 2013; Reichelt et al. 2013; Sevenster et al. 2013; Alfei et al. 2015), a finding that has also been incorporated by computational models (Osan et al. 2011; Gershman et al. 2017). However, with increasing nonreinforced stimulus reexposure, it is unlikely that there is a sufficient qualitative or quantitative change in the prediction error signal to explain the transition to the null point and beyond to the NMDA receptor-dependent extinction phase. Moreover, it is not obvious how the instantiation of prediction error-mediated learning, for example, within the Rescorla–Wagner rule (Rescorla and Wagner 1972), is consistent with the new learning that is characteristic of extinction, rather than prediction error-mediated memory weakening (Exton-McGuinness et al. 2015). Indeed, it is possible, and perhaps likely, that there are independent mechanisms controlling destabilization and NMDA-receptor dependent extinction (Exton-McGuinness et al. 2015). While a sharp transition between reconsolidation and extinction might suggest a direct

interaction between the two, the three-phase transition with an intermediate null point may reflect the independent control of reconsolidation and extinction. Indeed, it could also explain occurrences when there appears to be no competition between reconsolidation and extinction (Duvarci et al. 2006). In such cases, destabilization/reconsolidation might be triggered regardless of the extent of stimulus reexposure, and may even overlap with the engagement of extinction.

In conclusion, our results demonstrate that during the retrieval of contextual fear memories, there is a genuine "null point" between the parameters that induce reconsolidation and extinction, at which the memory is not sensitive to disruption by MK-801. Nevertheless, context reexposure can still lead to NMDA receptor-independent decreases in freezing during this null point. These findings reinforce and expand the hypothesis of a three-phase transition between reconsolidation and extinction of associative memories, bringing new insights on the different ways a mnemonic trace might be affected by memory retrieval.

Materials and Methods

Subjects

Subjects were 228 experimentally naïve adult male Lister Hooded rats (200–350 g at the start of the experiment) from Charles River (UK). Animals were housed in groups of four per cage, under a 12 h light–dark cycle (lights on at 07:00) and a 21°C temperature, with water and food provided ad libitum apart from during the behavioral sessions. Cages contained aspen chip bedding, and environmental enrichment was available in the form of a Plexiglas tunnel. Experiments took place in a behavioral laboratory between 10:00 and 14:00. At the end of the experiment, animals were humanely killed via a rising concentration of CO_2; death was confirmed by cessation of heartbeat. All procedures were approved by a local ethical review committee and conducted in accordance to the United Kingdom Animals (Scientific Procedures) Act 1986, Amendment Regulations 2012 (PPL 70/7662).

Behavioral apparatus

The conditioning chambers (MedAssociates) consisted of two identical illuminated boxes (25 cm × 32 cm × 25.5 cm), placed within sound-attenuating chambers. The box walls were constructed of steel, except by the ceiling and front wall, which were made of Perspex. The grid floor consisted of 19 stainless steel rods (4.8 mm diameter; 1.6 mm center to center), connected to a shock generator and scrambler (MedAssociates). Infrared video cameras were mounted on the ceiling of the chambers (Viewpoint Life Sciences) and used to record behavior.

Contextual fear conditioning

During the training session, rats were placed individually in the conditioning chambers. After 3 min of free exploration, animals received 2 footshocks (0.7 mA, 1.5 sec) separated by a 30-sec interval, and after 1 min, were placed back into their home cages. Two days later, animals were reexposed to the same context for 3, 5, 10, 20, or 30 min. One day later, animals were exposed one more time to the context for 3 min, in order to assess memory expression (test session). No footshock was applied at either reexposure or test sessions. The aversive response (freezing) was automatically quantified during all sessions with a videotracking software (Viewpoint Life Sciences), and used as a memory index (Lee and Hynds 2013; Song et al. 2016).

Elevated plus maze

A standard maze composed of two open arms and two closed arms from MedAssociates was used. The rats were placed individually in the center of the maze, facing an open arm, and allowed 10 min of free exploration. Time spent in the open arms was scored manually by a researcher based outside the experimental room, and used as an index for baseline anxiety. Animals were considered to be in the arm when all four paws were placed within (Hu et al. 2014).

Drugs

MK-801 (Sigma-Aldrich) was diluted in sterile saline (0.1 mg/mL) and injected intraperitoneally (1 mL/kg) immediately after the reexposure session, or 30 min previously when specified (Lee et al. 2006; Song et al. 2016). Injections of MK-801 or vehicle were randomly distributed between animals.

Statistics

Data were analyzed in JASP (JASP Team 2017). Between-group comparisons were performed with one-way or two-way ANOVA, where needed. For within-group comparisons, repeated-measures ANOVA was applied. Levene's test was used for comparison of variability between groups. For slope and interception comparisons of linear regressions, data were analyzed in Prism (GraphPad Software 2017). Significance was always set at $P < 0.05$ and data are presented as mean + SEM. Animals freezing more than 95% during the context reexposure sessions were excluded from analysis (five animals). The rationale for this was that asymptotic learning appears to result in a resistance to memory destabilization (Rodriguez-Ortiz et al. 2005, 2008; Lee 2010), and so rats that froze at near-maximal levels during context reexposure would be unlikely to undergo reconsolidation regardless of reexposure duration. Similarly, animals that do not learn at all would not be suited to detect reconsolidation or extinction impairments, and so a criterion of >5% freezing was also imposed, although this did not result in the exclusion of any animals. n_p^2 was used as an estimate of effect size and BF_{10} is also reported as the outcome of Bayesian analyses for the estimation of posterior probability (Jarosz and Wiley 2014).

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Supplemental Material

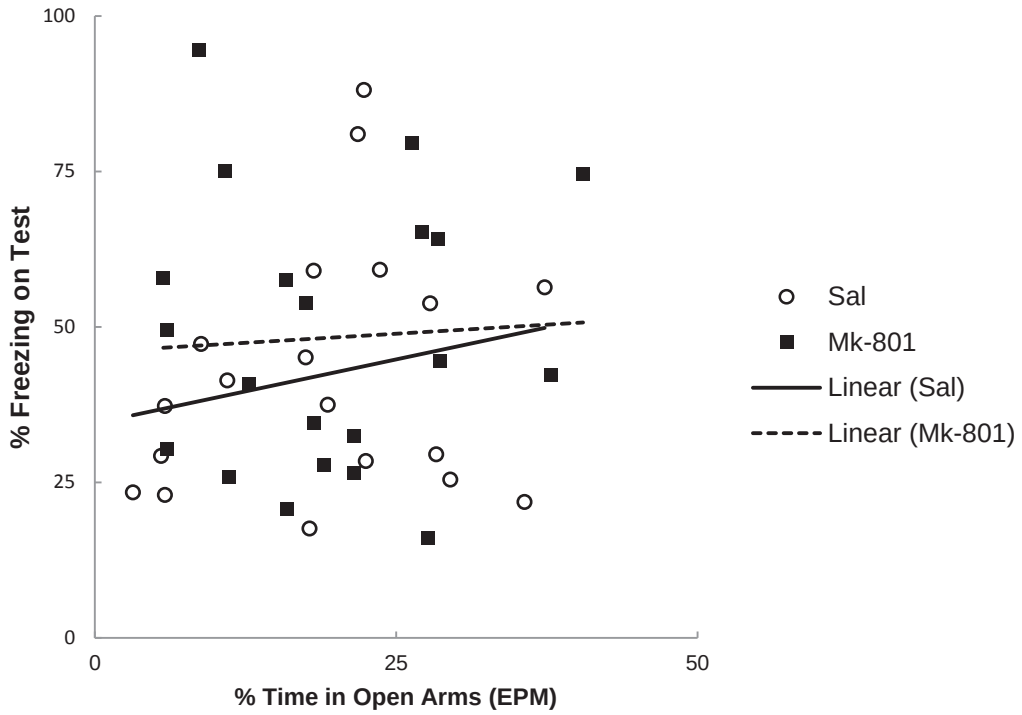


Fig. S1. Baseline anxiety does correlate with freezing during CFC test. Animals were placed individually in an Elevated plus maze (EPM) and allowed 10 min of free exploration. Time spent in the open arms was used as index for baseline anxiety and correlated against the percent time freezing during the contextual fear memory test. No correlation was observed in either of the groups ($p > 0.390$). Additional correlations are depicted in Figure 3. n = 19-21 per group.

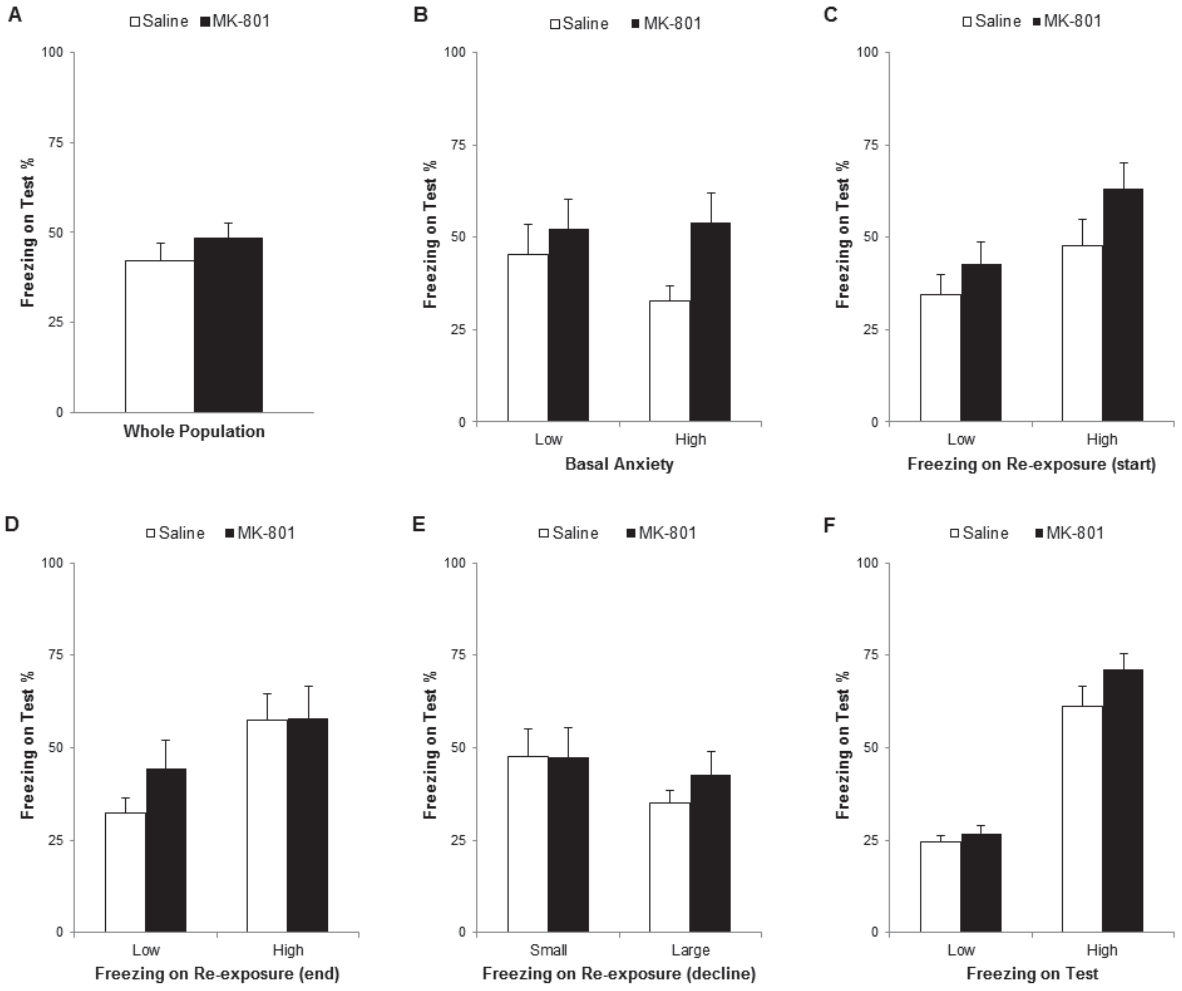


Figure S2. Subgroup analysis of animals undergoing 10-min context re-exposure. Animals were subjected to contextual fear conditioning in big cohorts and, two days later, to an intermediate re-exposure session of 10-min. Immediately after, they received i.p MK-801 or saline, and on the following day memory was assessed in a test session. Animals were then allocated to two different groups, according to the following parameters: basal anxiety, freezing at the start (first 3min) and at the end (last 3min) of the context re-exposure, freezing decline across re-exposure session (start – end) and freezing at test. For each parameter, values from subjects of each group (saline & MK-801) were ordered from smallest to largest. The top 8 animals were allocated to one group (e.g. low freezers) and the bottom 8 to the opposite (e.g. high freezers). Thereafter, the differential effect of MK-801 was analysed on these subpopulations. **(A)** MK-801 did not have any effect when analysing the population as a whole ($F_{1,38}$ = 0.85, p = 0.362; η^2_p = 0.02; BF_{10} = 0.43). Moreover, two-way ANOVA analysis revealed that the MK-801 effect did not depend upon **(B)** the baseline anxiety of animals (drug: $F_{1,28}$ = 3.76, p = 0.062, η^2_p = 0.12; BF_{10} = 1.39; subpopulation: $F_{1,28}$ = 0.56, p = 0.463, η^2_p = 0.02; BF_{10} = 0.41; drug x subpopulation: $F_{1,28}$ = 0.94, p = 0.340, η^2_p = 0.03; BF_{10} = 0.33). **(C)** The level of freezing during the start of the re-exposure session did not seem to be an important factor either (drug: $F_{1,28}$ = 3.33, p = 0.079, η^2_p = 0.11; BF_{10} = 0.98; subpopulation: $F_{1,28}$ = 6.72, p = 0.015, η^2_p = 0.19; BF_{10} = 3.39; drug x subpopulation: $F_{1,28}$ = 0.31, p = 0.582, η^2_p = 0.01; BF_{10} = 2.25). **(D)** There was also no effect of MK-801 regardless of the freezing level of individuals at the end of the re-exposure session (drug: $F_{1,28}$ = 0.81, p = 0.377, η^2_p = 0.03; BF_{10} = 0.43; subpopulation: $F_{1,28}$ = 7.73, p = 0.010, η^2_p = 0.22; BF_{10} = 5.61; drug x subpopulation: $F_{1,28}$ = 0.68, p = 0.416, η^2_p = 0.02; BF_{10} = 1.39). These analyses did, however, confirm that rats that froze more at the start or end of context re-

exposure also froze more at the subsequent test. **(E)** Furthermore, MK-801 did not have any effect no matter whether animals presented a small or a large decline of freezing across the re-exposure session (drug: $F_{1,28} = 0.34$, $p = 0.567$, $\eta^2_p = 0.01$; $BF_{10} = 0.38$; subpopulation: $F_{1,28} = 1.69$, $p = 0.204$, $\eta^2_p = 0.06$; $BF_{10} = 0.66$; drug x subpopulation: $F_{1,28} = 0.38$, $p = 0.542$, $\eta^2_p = 0.01$; $BF_{10} = 0.12$). **(F)** Finally, considering individual differences during the test itself, we also observed that MK-801 exerted no effect upon memory either on high- or low-freezing animals (drug: $F_{1,28} = 2.66$, $p = 0.114$, $\eta^2_p = 0.09$; $BF_{10} = 0.41$; subpopulation: $F_{1,28} = 117.42$, $p < 0.001$, $\eta^2_p = 0.81$; $BF_{10} = 3.79$; drug x subpopulation: $F_{1,28} = 1.12$, $p = 0.299$, $\eta^2_p = 0.04$; $BF_{10} = 1.92$). There was no evidence for MK-801 either impairing reconsolidation to reduce freezing below the level of equivalent subpopulation control animals, or disrupting extinction to increase freezing above the level of equivalent controls. Therefore, none of these analyses support the hypothesis that there are inter-individual differences in the response to MK-801, and instead are more consistent with the existence of a null point at the individual level. Data are presented as mean + SEM. n = 19-21 per group / 8 per subgroup.

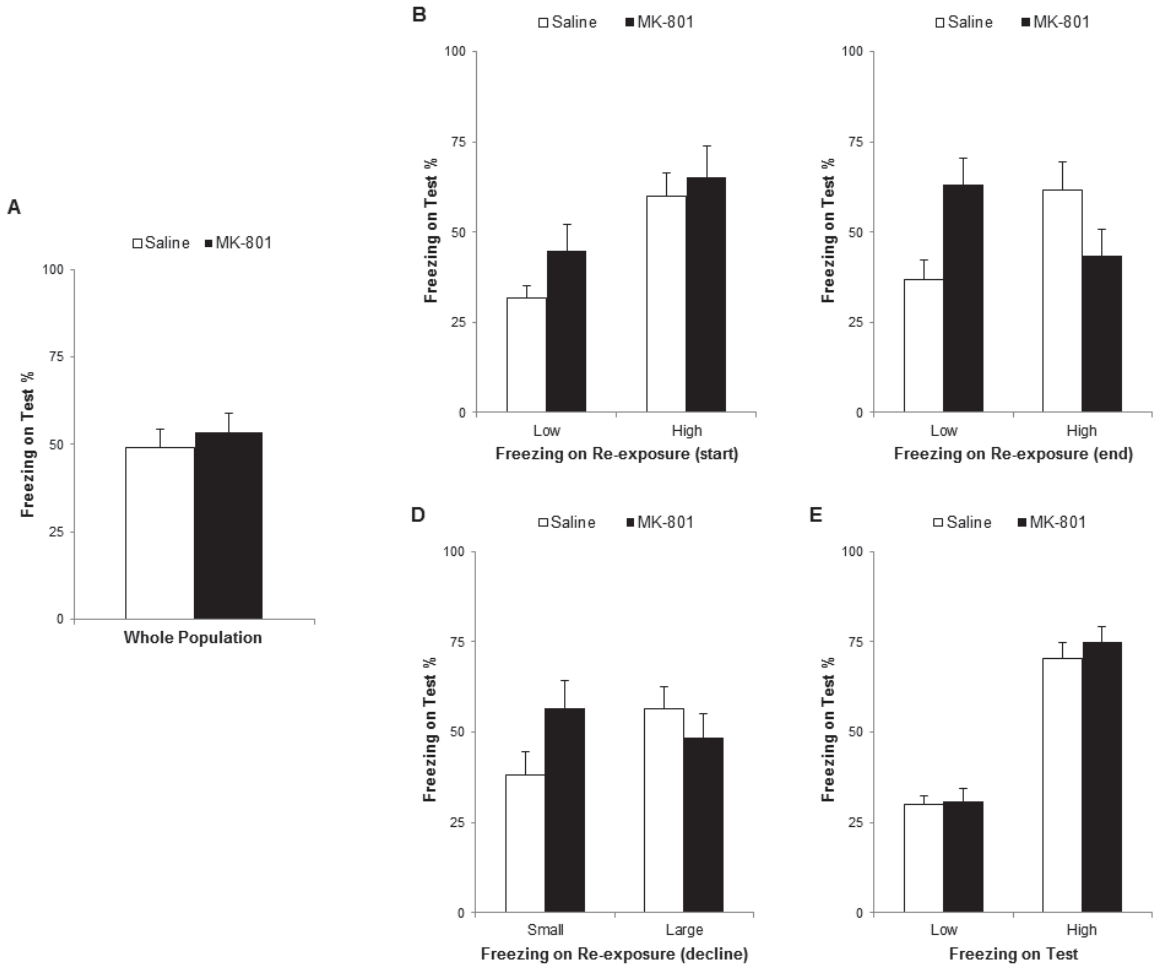


Figure S3. Subgroup analysis of animals undergoing 5-min context re-exposure. Animals were subjected to contextual fear conditioning in big cohorts and, two days later, to an intermediate reactivation session of 5-min. Immediately after, they received i.p MK-801 or saline, and on the following day memory was assessed in a test session. Animals were then allocated to two different groups, according to the following parameters: freezing at the start (first 3min) and at the end (last 2min) of the context re-exposure , freezing decline across context re-exposure (start – end) and freezing at test. For each parameter, values from subjects of each group (saline & MK-801) were ordered from smallest to largest. The top 8 animals were allocated to one group (e.g. low freezers) and the bottom 8 to the opposite (e.g. high freezers). Thereafter, the differential effect of MK-801 was analysed on these subpopulations. **(A)** MK-801 did not have any effect when analysing the population as a whole ($F_{1,36} = 0.35$, $p = 0.558$; $\eta^2_p = 0.01$; $BF_{10} = 0.36$). Moreover, two-way ANOVA analysis revealed that the MK-801 effect did not depend upon **(B)** the level of freezing during the start of the re-exposure session (drug: $F_{1,28} = 1.75$, $p = 0.197$, $\eta^2_p = 0.06$; $BF_{10} = 0.55$; subpopulation: $F_{1,28} = 12.34$, $p = 0.002$, $\eta^2_p = 0.31$; $BF_{10} = 23.03$; drug x subpopulation: $F_{1,28} = 0.31$, $p = 0.582$, $\eta^2_p = 0.01$; $BF_{10} = 6.87$). **(C)** There was a significant interaction between drug and subgroup based on the level of freezing at the end of re-exposure (drug: $F_{1,28} = 0.32$, $p = 0.577$, $\eta^2_p = 0.01$; $BF_{10} = 0.37$; subpopulation: $F_{1,28} = 0.14$, $p = 0.714$, $\eta^2_p = 0.01$; $BF_{10} = 0.35$; drug x subpopulation: $F_{1,28} = 9.85$, $p = 0.004$, $\eta^2_p = 0.26$; $BF_{10} = 1.45$). However, this interaction is likely not meaningful as it is also seen at the start ($F_{1,28} = 13.90$, $p < 0.001$, $\eta^2_p = 0.33$; $BF_{10} = 14.82$) and at the end ($F_{1,28} = 21.97$, $p < 0.001$, $\eta^2_p = 0.44$; $BF_{10} = 172.42$) of re-exposure , probably reflecting a pre-existing difference rather than an MK-801-induced difference. **(D)** Furthermore, MK-801 did not have any effect no matter whether

animals presented a small or a large decline of freezing across the re-exposure session (drug: $F_{1,28}$ = 0.57, p = 0.455, η^2_p = 0.02; BF_{10} = 0.41; subpopulation: $F_{1,28}$ = 0.55, p = 0.466, η^2_p = 0.02; BF_{10} = 0.41; drug x subpopulation: $F_{1,28}$ = 3.76, p = 0.063, η^2_p = 0.12; BF_{10} = 0.25). **(E)** Finally, considering individual differences during the test itself, we also observed that MK-801 exerted no effect upon memory either on high- or low-freezing animals (drug: $F_{1,28}$ = 0.49, p = 0.490, η^2_p = 0.02; BF_{10} = 0.35; subpopulation: $F_{1,28}$ = 119.95, p < 0.001, η^2_p = 0.81; BF_{10} = 1.51; drug x subpopulation: $F_{1,28}$ = 0.25, p = 0.618, η^2_p = 0.01; BF_{10} = 2.69). There was no reliable evidence for MK-801 either impairing reconsolidation to reduce freezing below the level of equivalent subpopulation control animals, or disrupting extinction to increase freezing above the level of equivalent controls. Therefore, these analyses do not support the hypothesis that there are inter-individual differences in the response to MK-801, and instead are more consistent with the existence of a null point at the individual level. Data are presented as mean + SEM. n = 19 per group / 8 per subgroup.

Chapter IV

ON THE SYNAPTIC TAGGING AND CAPTURE HYPOTHESIS

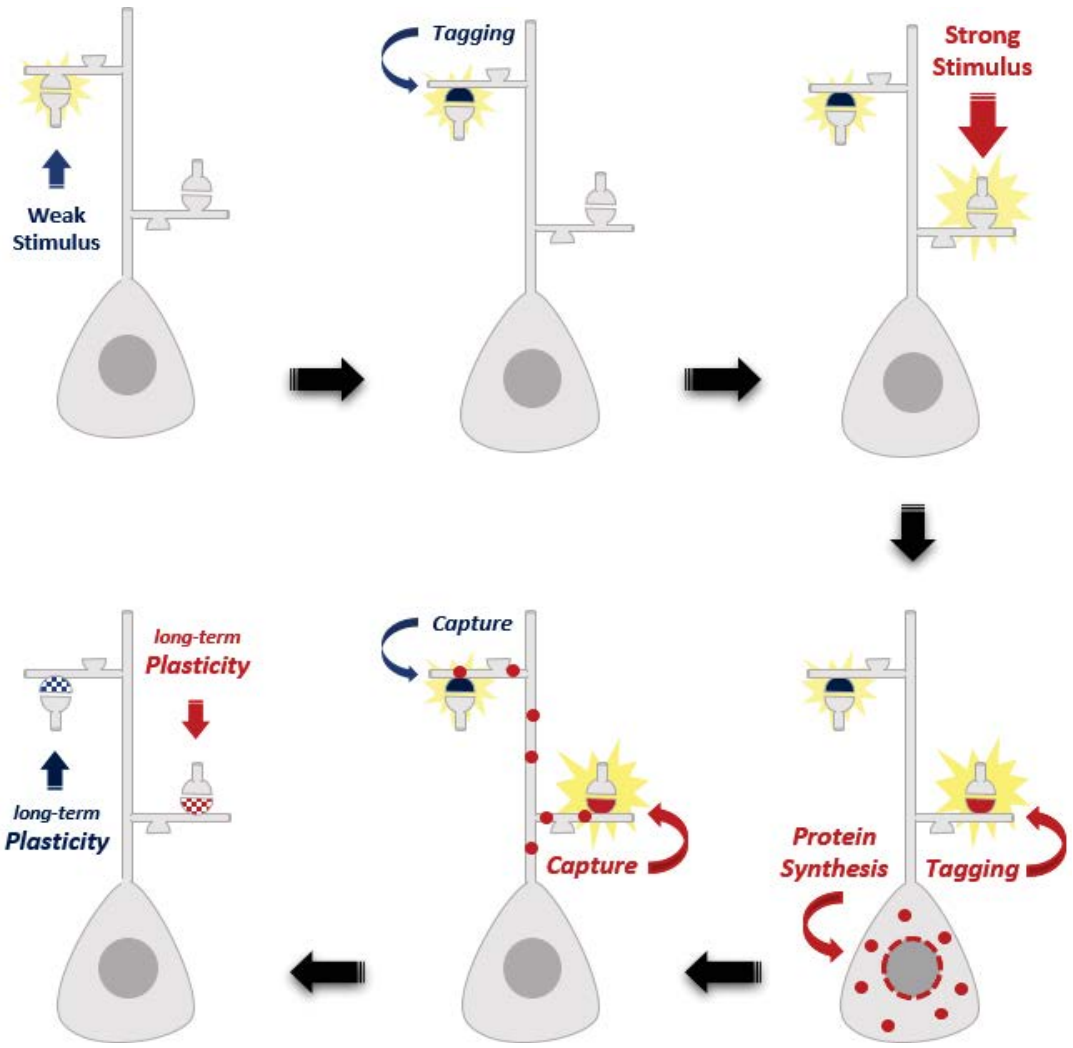
4.1 Introduction

The establishment of a memory at the cellular level, known as cellular consolidation, has long being related to long-term modifications in neuronal connectivity, mediated by synaptic plasticity and protein synthesis (Rosenberg *et al.*, 2014; Dudai, Karni and Born, 2015; Sweatt, 2016). One very important characteristic of synaptic plasticity is it specificity, that is, when a given neuron is stimulated, not all synaptic connections present within the cell will go through modifications, but only those involved and active during stimulation. The synthesis of new plasticity related proteins, which are essential for the stabilization and maintenance of long-term synaptic modifications, requires the expression of genetic material though, which can be found in the neuronal soma and dendrites, but not synapses. Therefore, it remained unclear how proteins synthetized in the neuron are able to sustain plasticity only and specifically on the synapses previously stimulated. The phenomenon described in electrophysiology as Synaptic Tagging and Capture (STC) has been proposed by Frey and Morris in 1997 to address this issue, and may add important details on the mechanisms and properties of memory consolidation (Redondo and Morris, 2011).

The STC proposes the strong stimulation of a synaptic pathway can lead to the following, distinguishable events: (1) synaptic *tagging*, a process by which the stimulated synapses become specifically and temporally permissive to plastic modifications and (1b) synthesis of plasticity-related proteins (PRPs) in the cell nucleus and dendrites. PRPs are then (2) *captured* by the tagged synapses, allowing the establishment of long-lasting modifications in the strength and efficiency of the stimulated synapses. If PRPs are not available during tagging, either by insufficient stimulation or pharmacological manipulation, the receptive state of the synapses will fade away and return to its normal baseline state (Frey and Morris, 1997; Barco et al., 2008;

Redondo and Morris, 2011). Interestingly though, if a weak stimulation is at least able to induce synaptic *tagging*, we could expect PRPs produced by a separate and stronger synaptic stimulation to be captured and used to sustain synaptic plasticity for both weak and strong stimulations, as long as neuronal activity coincides in space and time. That is, if both events recruit similar neuronal population, and hold active *tagged* synapses by the time PRPs become available, the STC hypothesis predicts an *association phenomenon* that would allow the establishment of long-term plasticity for both events indistinguishably (Fig. 4.1).

Figure 4.1



The association phenomenon in the STC hypothesis. Schematic representation.

The STC hypothesis has been vastly tested and validated in cellular electrophysiology since 1998, when first proposed by Frey and Morris (Frey and Morris, 1998). In 2007, the concept was then first tested at the level of behaviour by Moncada and Viola in a study that associated two hippocampus-dependent learning experiences (Moncada and Viola, 2007). First, male Wistar rats were submitted to weak training in the inhibitory avoidance (IA) paradigm. During training, rats were placed on a small platform within a metal-grid floor chamber, and as they stepped-down, a weak footshock was applied (0.15 mA, 2 s). When placed again on the platform, shortly after training (15 min), animals were observed to express short-term memory (STM) by showing an increased step-down latency. However, training was not sufficient to result in long-term memory (LTM), as shown by test realized 24h later. Next, the weak training was associated with exploration to a novel Open Field (OF) arena, in order to induce strong hippocampus-dependent learning, at several times before and after training. In accordance to the STC hypothesis, a time-dependent association phenomenon was observed, allowing LTM formation for the same training, which, otherwise, would only induce STM. The phenomenon was shown to depend on new protein-synthesis resulting from the novel OF experience, but not from the IA learning itself, indicating the occurrence of an association phenomenon mediated by synaptic tagging and capture mechanisms. Additionally, the same phenomenon was observed when a strong training in the inhibitory avoidance was used, preceded shortly by the administration of anisomycin, a protein synthesis inhibitor, or its vehicle in the hippocampus. Animals treated only with vehicle exhibited in this case robust LTM when tested 24h later, which was abolished by the injection of anisomycin. As predicted by the STC hypothesis, though, exposure to a novel OF one hour before strong training on the IA plus anisomycine injection, allowed the formation of LTM in spite of the amnesic treatment.

In 2009, Ballarini and colleagues reinforced and expanded the STC concept by employing both hippocampal and non-hippocampal, as well as aversive and non-aversive, memory paradigms. (Ballarini et al., 2009). First, animals were subjected to a weak training able to induce STM, but not LTM, in the spatial object recognition (SOR) task. During training, animals were placed in an experimental arena containing spatial clues and two identical objects, for which exploration time was quantified. Thereafter, 30min or 24h, one of the objects was relocated to a different position and animals were placed again in the arena. Memory was then assessed by the ability of animals to recognize the change on the spatial configuration, expressed as preferential exploration of the repositioned object. Animals only submitted to the weak SOR training did not express any significant LTM for the experience. However, when the event was associated with exploration of a novel open field (OF), one hour before or one hour after training, animals were then observed to express relevant SOR-LTM for the weak experience. Again, the phenomenon was not observed when protein synthesis was inhibited with intra-hippocampal anisomycin administered close to the novel, but not the weak, experience. The same association was then observed when weak training in the contextual fear conditioning (CFC) paradigm (discussed previously on chapters 1 and 2) was also associated with novel OF and hippocampal anisomycin administration. Next, the phenomenon was shown to not be exclusively related to hippocampal-dependent learning, suggesting the synaptic tagging and capture may be a general mechanism involved in memory formation. For that, rats were submitted to weak training in the conditioned taste aversion (CTA) paradigm, which requires the activation of the insular cortex (Bermudez-Rattoni, 2004). In this task, rats associate the consumption of saccharin with the i.p. injection of a lithium chloride (LiCl) solution, which causes digestive distress. Animals then expressed STM for the event by significantly avoiding the consumption of saccharin when submitted to a test session shortly after training, but no

LTM was observed in a test performed three days after. Nevertheless, when a new gustative stimulus, supposedly able to activate and induce strong learning in the insular cortex, was associated with the weak CTA training, an association phenomenon was again observed and blocked when anisomycin was administered locally in the correspondent brain region. Interestingly, the same phenomenon was not observed when taste aversion was associated with novel spatial information (OF), nor when weak training in the SOR paradigm was associated with novel gustatory information, further supporting the occurrence of a STC-mediated phenomenon, which required both weak and strong events to coincide in time and space for both to persist.

On the last eleven years, the associative phenomenon described by Viola and colleagues has been replicated in many other studies employing several behavioural, pharmacological and molecular approaches. These studies are summed up in Table 4.1. and together offer a comprehensible evidence for the involvement of a *synaptic tagging and capture* mechanism in the consolidation of memory.

Table 4.1

Year	Author	PRPs "donator" experience		PRPs "receptor" experience		Pharmacological Tools		Other Tools
		Task	Variations	Task	Variations	Drugs	Infusion	
2007	Moncada & Viola	Open Field	Novel Familiar	Inhibitory Avoidance (*step down*)	Weak Training Strong Training	Anisomycin SCH23390	Hippocampus	-
2009	Ballarini et al.	Open Field Taste	Novel Familiar	Spatial Object Recognition Contextual Fear Conditioning Conditioned Taste Aversion	Weak Training	Anisomycin	Hippocampus Insular Cortex	Quantification of pCREB
2010	Wang et al.	Open Field	Novel	"Everyday" Spatial Memory	Weak Training Strong Training	Anisomycin SCH23390	Hippocampus	Electrophysiology Meta-analyse
2011	Moncada et al.	Open Field	Novel	Inhibitory Avoidance (*step down*)	Weak Training Strong Training	AP5 Anisomycin Emetine KN62 Propranolol Rp-cAMP SCH23390 U0126 Dobutamine SKF-38393	Hippocampus Intraperitoneal	-
2011	Lu et al.	Open Field	Novel	Inhibitory Avoidance (*step through*)	Weak Training	1NMPP1	Intraperitoneal	TrkB 1NMPP sensitive mutants Electrophysiology

2012	Almaguer-Melian et al.	Open Field	Novel Familiar	Morris Water Maze	Strong Training	Anisomycin	Hippocampus	Stress Quantification of Arc and BDNF
2013	Myskiw et al.	Open Field	Novel Familiar	Contextual Fear Conditioning	Weak Extinction	Anisomycin DRB Rapamycin	Hippocampus Amygdala	-
2013	Cassini et al.	Re-exposure to Fear Conditioned Context Re-exposure to Water Maze Context	Retrieval Only Reconsolidation Extinction Reconsolidation	Spatial Object Recognition	Weak Training Very Weak Training	AP5 Cycloheximide D-Cycloserine Nimodipine	Hippocampus Intraperitoneal Subcutaneous	-
2013	Ballarini et al. **(in humans)**	Science class Music Lesson	Novel Familiar	Literary Memory Study Complex Figure Test	Weak Training	-	-	-
2014	Salvetti et al.	T-maze reward task Open Field Object Recognition	Home cage, same, increased, delayed, and different reward Novel/Familiar -	"Everyday" Spatial Memory	Weak Training	-	-	-
2014	Myskiw et al.	Open Field	Novel	Contextual Fear Conditioning	Weak Extinction	Anisomycin AIP AP5 β-lactacystin Nifedipine Rapamycin	Hippocampus	-
2015	Menezesa et al.	Open Field	Novel	Inhibitory Avoidance (*step down*)	Weak Extinction	Anisomycin SCH23390 Rp-cAMP Gö6976 Sp-cAMP PMA	Hippocampus	HPLC for dopamine levels in hippocampus

Table 4.1. Resume of published studies on the STC hypothesis and memory.

Once consolidated, memories that are initially unstable and labile, are brought to a stable state which is no longer susceptible to interference and is capable of maintaining memory for days, weeks, or even months (Katche, Cammarota and Medina, 2013). Under certain circumstances though, consolidated memories can still return to a plastic state, through a process known as destabilization or labilization. This phenomenon allows memories to incorporate important modifications that may come to be relevant, such as updating and strengthening (de Oliveira Alvares et al, 2012, 2013; Lee, 2010, 2008). However, once labilized, memories become once again unstable and susceptible to disruption, and will require the re-stabilization of any new and previous synaptic modifications in order to persist. This process is known as reconsolidation and also dependents on protein synthesis (Suzuki et al., 2004; Tronson and Taylor, 2007; Dudai, 2012; Nadel et al., 2012).

Considering that both consolidation and reconsolidation requires the synthesis of plasticity related proteins, and that enough evidence suggests memory consolidation is related to STC mechanisms, we hypothesised that memory destabilization and reconsolidation could, similarly, involve a process of synaptic (re)-tagging and capture. Reactivation of a previous memory could induce a process of synaptic (re)-tagging, bringing the involved synaptic connections to a new and receptive plastic state, that would catheterize the process we know as labilization/destabilization of the memory. Parallelly, new PRPs would then be produced in the soma and dendrites of the neuronal network and "captured" by the tagged synapses in order to allow the reconsolidation of the memory, through the re-stabilization of the previous, and potentially new synaptic modifications.

If true, we would expect for the association phenomenon observed during the consolidation of newly acquired memories to also be observable during the process of memory reconsolidation. In this case, the administration of amnesic agents that would normally impair reconsolidation and lead to amnesia, could hypothetically not lead to any significant disruption if associated with an independent strong experience, capable of inducing protein synthesis, if both events are close in time and share a similar neuronal population. To test that, we associated the reactivation of a contextual fear memory and the administration of MK-801 with a new learning experience, and evaluated whether memory remained undisturbed, or not, after treatment.

4.2 Methods

Subjects: Subjects were 15 experimentally naïve adult male Lister Hooded rats, weighing 200–350 g at the start of the experiment, from Charles River (UK). Animals were housed in groups of four per cage, under a 12 h light–dark cycle (lights on at 07:00) and a 21°C temperature, with water and food provided ad libitum, apart from during the behavioural sessions. Cages contained aspen chip bedding, and environmental enrichment was available in the form of a Plexiglas tunnel. Experiments took place in a behavioural laboratory between 10:00 and 14:00. At the end of the experiment, animals were humanely killed via a rising concentration of CO_2; death was confirmed by cessation of heartbeat. All procedures were approved by a local ethical review committee and conducted in accordance to the United Kingdom Animals (Scientific Procedures) Act 1986, Amendment Regulations 2012 (PPL 70/7662).

Contextual fear conditioning: The behavioural procedure consisted of training, reactivation, and test sessions, as previously described (Cassini *et al.*, 2017). During sessions, rats were transported in their homecages to a brightly illuminated room, where the conditioning chambers were located. Afterwards, animals were individually placed in the chambers and after 3 min of freely context exploration, received 2 footshocks of 0.7 mA (1.5 sec, 30-sec interval). Animals then remained in the chamber for one additional minute before being placed back in their homecages and transported to the holding room (training session). Two days later, animals were again transported to the same room and reexposed to the conditioning chambers for 3 min (reactivation session). Three minutes reexposure has been shown before, under the same experimental conditions, to induce memory destabilization (Cassini et al. 2017). On the next day, animals were exposed once more to room and chambers, for 3 min, in order to access memory expression (test session). No footshock was applied at either reactivation or test sessions. The aversive behaviour in response to the conditioned context (freezing), was automatically quantified during all sessions with videotracking software (Viewpoint Life Sciences) and used as memory index (Lee and Hynds 2013; Song et al. 2016).

Open Field (OF): The OF apparatus consisted of square arena with black plywood floor and walls, which contained different visual clues (Fig. 4.2). The arena was located in a separate room, illuminated by indirect and reduced light, and procedures were carried out by a different experimenter. In addition, animals were transported to the room individually in transport boxes instead of the homecages. This was done in order to maintain certain independence between the two events, since too much similarity could potentially lead to memory interference and not association (Moncada and Viola, 2007). Moreover, it was important to keep the two experiences as two independent events, and not one single incident, to reliably replicate original STC electrophysiological experiments. Animals were then placed in the arena and allowed to

explore the new environment for 5 min, before being transported back to the animal room. One hour later, animals were than submitted to the fear reactivation session described before.

Figure 4.2

Picture of arena used for the novel experience. Top View.

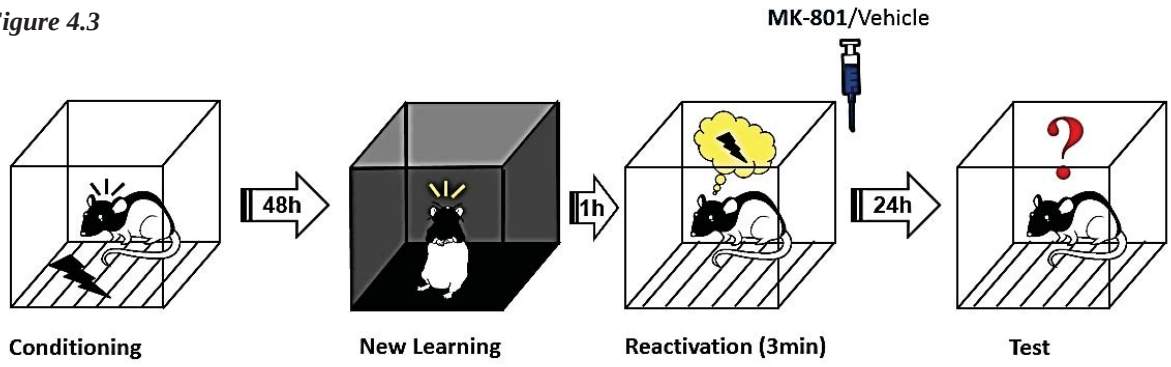
Drugs: MK-801 (Sigma-Aldrich) was dissolved in sterile saline (0.1 mg/ml) and injected intraperitoneally (1 ml/kg) immediately after the fear reactivation session (Cassini et al., 2017, Flavell & Lee., 2013, Merlo et. al., 2014). Injections of MK-801 or vehicle were randomly allocated between animals accordingly to order generated with List Randomizer (https://www.random.org/lists).

Statistics: Data were analysed in SPSS (IBM Corp, 2015). Two-way ANOVA was used to analyse effects of between-groups factors, or repeated-measures ANOVA when within-group factor was present. Further analysis of simple main effects was performed with one-way ANOVA. Significance was set at $p < 0.05$ and data are presented as mean + SEM. As an estimate of effect size, n_p^2 was used. Animals freezing more than 95% or less than 5% during reactivation were excluded from analysis (1 and 0 animals, respectively) as described before (Cassini et al., 2017).

4.3 Results

Lister-Hooded rats were subjected to a pilot experiment consisting of contextual fear conditioning (CFC) on day 1, short reactivation on day 3 followed by the intraperitoneal administration of MK-801 (0.1 mg/kg) or Saline, and test on day 4. MK-801 has been previously shown to impair memory reconsolidation under the exactly parameters used here (Cassini et. al., 2017) and similar experimental conditions (Lee et al. 2006; Flavell & Lee 2013; Merlo et al. 2014). The aversive response (freezing) was automatically recorded during all sessions and used as an index of fear memory. In order to test whether the amnesic effect of MK-801 could be rescued by the association of a concomitant learning experience, which would indicate underlying synaptic tagging and capture processes, animals were exposed to a novel Open Field (OF) arena one hour before reactivation of the fear memory and the drug administration (Fig. 4.3). Exploration of an unfamiliar Open Field has been shown before to be related with protein synthesis in the hippocampus (Kerr, Beck and Handa, 1996; Vianna et al., 2000; Martínez et al., 2012) and to support memory consolidation of diverse tasks performed one hour before or after the novelty exposure (Moncada and Viola, 2007; Ballarini et al., 2009; Almaguer-Melian et al., 2012; de Carvalho Myskiw, Benetti and Izquierdo, 2013; Salvetti, Morris and Wang, 2014).

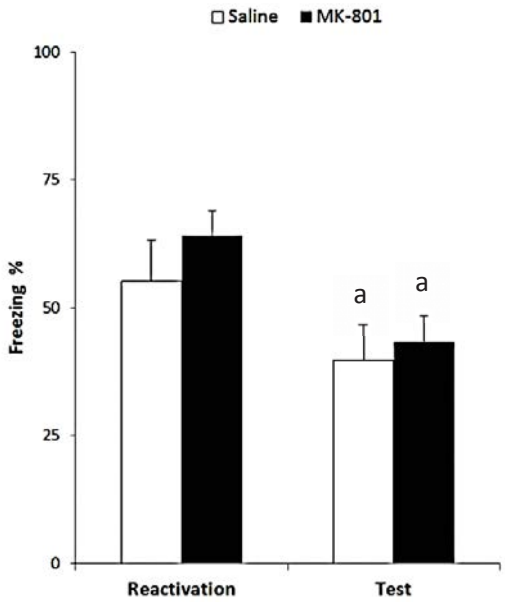
Figure 4.3



Experimental design.

Figure 4.4a shows expression of contextual fear in animals that received MK-801 after a short reactivation session, preceded by a novel experience (open field). Freezing between groups (saline x MK-801) did not differ either during reactivation ($F_{1,15}$ = 0.91, p = 0.356) or test sessions ($F_{1,15}$ = 0.18, p = 0.674; n = 7-8 per group). The absence of significant differences suggests the new learning was able to overcome the expected amnesic effect of MK-801, which we observed before with 3min reactivation session (Cassini et al., 2017). Moreover, the consistent freezing reduction observed between sessions on Chapters I and II, was again observed here with both vehicle ($F_{1,7}$ = 15.29, p = 0.006; n^2_p = 0.69; n = 7) and MK-801 ($F_{1,7}$ = 6.90, p = 0.034; n^2_p = 0.50; n = 8) groups (Fig. 4.4b), indicating that the additional experience *per se* did not affect memory processing. These observations were additionally confirmed with repeated-measures ANOVA, which revealed a main effect of session ($F_{1,14}$ = 16.76, p = 0.001, n^2_p = 0.54) and no effect of drug ($F_{1,14}$ = 0.67, p = 0.426) or drug x session interaction ($F_{1,14}$ = 0.37, p = 0.552).

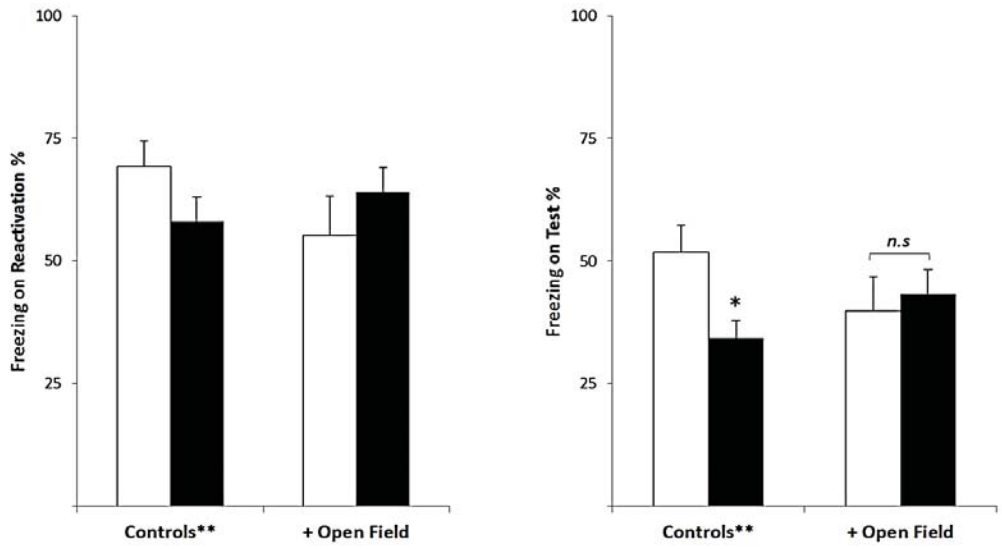
Figure 4.4



Percent freezing on reactivation and test sessions of animals exposed to a novel experience. It has been no significant difference between groups (saline x MK-801) on either reactivation or test sessions. There was, however, significant difference bewteen sessions (react x test) on both saline and MK-801 groups. [a] p <0.05. Data presented as mean + SEM. n = 7 (saline) and 8 (MK-801).

Next, we compared the results found here with the equivalent control condition (no novelty) described on Chapter III, for exploratory purposes only. When analysing memory expression during the test session (Fig. 4.5b), two-way ANOVA revealed no main effect of novelty ($F_{1,43}$ = 0.07, p = 0.797) or drug ($F_{1,43}$ = 1.70, p = 0.200), but a nearly significant drug x novelty interaction ($F_{1,43}$ = 3.83, p = 0.057), indicating a probable differential effect of drug within different reactivation conditions (control x novelty).

Figure 4.5



*Percent freezing on **a)** reactivation and **b)** test sessions of animals exposed to a novel experience in comparisson to control condition:no novelty – open field before reactivation session (3 min) followed by saline or MK-801 injections. Data presented as mean + SEM. n.s.: non significant. * p <0.05. **<u>Data from previous experiment presented on chapter III, added here for especulative purposes only</u>.*

However, during reactivation there was also a relatively close to significant interaction between drug and novelty ($F_{1,43}$ = 2.91, p = 0.096). This may suggest the tendency for interaction observed during test, instead of indicating a possible differential effect of drug within the novelty condition, may have been a result of pre-existing differences on the groups, since drug at that moment have not yet been administered (Fig. 4.5a). Nevertheless, there was no main effect of drug ($F_{1,43}$ = 0.04, p = 0.848), as would be expected, and no main effect of novelty either ($F_{1,43}$ = 0.48, p = 0.491), indicating the novel experience, which preceded the reactivation session, did not itself affect the expression and reactivation of the fear memory.

4.4 Discussion

In this study, we have observed that the administration of MK-801 after contextual fear reactivation had no effect upon reconsolidation when animals were previously exposed to a novel experience (Open Field). MK-801 has been demonstrated before to impair memory under this (Cassini et. al., 2017) and similar experimental conditions (Lee et al. 2006; Flavell & Lee 2013; Merlo et al. 2014), indicating that reconsolidation here has been supported by the new learning experience. This association phenomenon suggests that the process of memory destabilization and reconsolidation may involve mechanisms of *synaptic tagging and capture*.

It is very important to notice though that these results are only preliminary, and do not offer enough evidence for the involvement of STC mechanisms in the process of memory reconsolidation. For more comprehensive evidence, additional experimentation replicating and expanding the results presented here would be necessary. Primary, the experiment discussed on Figure 3.4 should be repeated, with all the appropriated control groups included (no novelty + saline / no novelty + MK-801), to reduce the impact of uncontrolled factors that could result

in the wrong rejection or acceptation of the null hypothesis. For example, the absence of MK-801 effect on animals exposed to novelty before memory reactivation could have resulted from a defective batch of the drug, problems with the administration, differential responsiveness on the batch of animals, both to the drug as to the contextual fear conditioning, etc. By running simultaneously all the experimental groups, factors like that would not necessarily be avoided, but more controlled and normalized between the experimental conditions. Additionally, a bigger sample size should also be considered in advance, in order to reduce variability and provide enough power for the detection or rejection of significant effects, since statistical analysis would involve multiple comparisons and variables (two-way ANOVA) (Wilson Van Voorhis and Morgan, 2007). If the preliminary results presented here are validated, the phenomenon behind the effects observed could then be further explored and better understood.

According to our hypothesis, the absence of MK-801 effect on animals previously exposed to a new learning resulted from an association phenomenon mediated by STC mechanisms. That is, when animals were exposed to the open field arena, we expect a new learning to have been induced, since similar conditions were show before to lead to long-term memory formation in the hippocampus (Vianna, 2000). The new learning at the cellular level, as we discussed before, is believed to bring the involved synaptic connections to a permissive state (*tagging*), and to induce the synthesis of plasticity related proteins (*PRPs*) (Ballarini *et al.*, 2009). Once the contextual fear memory is reactivated, one hour later, the connections encoding this memory would become permissive as well (second *tagging*), and the synthesis of PRPs would be induced again, in order to sustain the reconsolidation of the reactivated memory. On animals treated with MK-801 though, protein synthesis would supposedly be impaired, since the induction of it depends on NMDA activity (Pedreira, Pérez-Cuesta and Maldonado, 2002), leading to the amnesia we observe when reactivation and MK-801 are the only factors involved (Fig. 4.5). On those animals that have been exposed to a novel learning before, though, we

could expect for PRPs to already be available in the network, when animals receive MK-801. Considering both experiences rely on hippocampus plasticity (Vianna, 2000; Gafford, Parsons and Helmstetter, 2011), and that experiences close in time are more probable to share a common neural population (Zhou *et al.*, 2009), we could expect some level of overlapping between the two networks. If that is true, the PRPs produced in the neuronal soma in response to the novel learning, that preceded the contextual fear reactivation and drug administration, would be able to act and induce sustainable plastic modification on the tagged synapses involved in both, the novel learning and the contextual fear conditioning, allowing for the consolidation and reconsolidation of both events.

To test whether the phenomena observed was mediated by the mechanisms we propose here, in addition to the replication experiment it remains necessary to demonstrate whether the exposure to the novel open field induced the synthesis of plasticity related proteins in the hippocampus, and long-term memory; whether the reactivation of the contextual fear memory followed by MK-801 injection resulted in reduced protein synthesis in the same brain area, and, finally, whether both experiences activated similar neural population in the hippocampus when associated. Long-term memory for the open field can easily be assessed by analysing habituation behaviour on animals when returning to the arena. This can be achieved by comparing the extend of animal's navigation during first exposure to the arena, and during reexposure realized twenty-four hours later, or more (Vianna, 2000). Protein synthesis in the hippocampus could be assessed with techniques like western blotting, to quantify plasticity related products such as ArC (activity-regulated cytoskeleton-associated protein) (Lonergan *et al.*, 2010), and pharmacologically, with the administration of protein synthesis inhibitors, such as anisomycin, in the hippocampus (Naghdi, Majlessi and Bozorgmehr, 2003). Finally, the neural population activated during each experience could be assessed with the multi-labelling technic described in Zaidi et al. (2000), which permits the identification and differentiation of

neural cells activated by two distinct events. It would also be important to replicate the association phenomenon with post-reactivation, instead of pre-reactivation, exposure to novelty. Since novelty has been associated with dopamine release (Moncada *et. al.*, 2011), and release of dopamine is associated with the generation of prediction error (Reichelt, Exton-McGuinness and Lee, 2013), which can trigger memory destabilizaztion, it is possible the exposure to novelty before reactivation of the contextual fear memory had some influence on the memory destabilization. Altough we did not observe behaviourally any indication of an influence from the novel experience *per se* upon memory reactivation, an interaction remains plausible, and additional experimentation would be necessary to investidate de possibility. Moreover, replication of the phenomenon with the different timing of novelty exposure would reinforce the involviment of STC mechanisms on the phenomenon, since a time window of associativity extending both before and after an event is in accordance with the proposed mechanisms (Frey and Morris, 1997; Barco et al., 2008; Redondo and Morris, 2011).

In addition to the association phenomenon, manipulating synaptic tagging processes during memory reactivation would be useful as well to test whether memory reconsolidation involve STC mechanisms. According to our hypothesis, preventing synapses from entering a permissive state susceptible to modification, that is, *tagging*, should prevent memories from entering destabilization, protecting memory from interreference and disruption. Therefore, drugs as Latrunculin or KN-93, which have been shown to impair the process of *tagging* in electrophysiology (Ramachandran and Frey, 2009; Redondo *et al.*, 2010), should prevent the expected amnesic effects of protein synthesis inhibitors, such as anisomycin (Rodriguez-Ortiz *et al.*, 2008) and rapamycin (Gafford, Parsons and Helmstetter, 2011). Similarly, interfering with the tagging process during reactivation should protect memory from the disruptive effect of behaviour manipulations, such as presentation of distractor stimuli (Crestani *et al.*, 2015), appetitive information (Haubrich *et al.*, 2015) and extinction training (Monfils *et al.*, 2009), as

well as preventing processes as strengthening, updating and precision-keeping from supporting and enhancing memory reconsolidation (De Oliveira Alvares *et al.*, 2013). On the other side, facilitating mechanisms involved in synaptic tagging could allow the destabilization of memories that can be very resistant to disruption, such as remote, traumatic and drug-related memories (Frankland *et al.*, 2006; Brown *et al.*, 2008; Wood *et al.*, 2015).

Although our results here are preliminary and require several additional experiments for validation, some evidence supporting our proposal has been recently published in the literature (Wang, 2018). Aiming to support the persistence of previous acquired weak memories, Wang associated a reactivation session with novelty exposure (open field arena), in a rodent appetitive spatial paradigm. Novelty was observed to prevent the normal decay of the weak memory on the course of 24h, however, it was not clear whether the effect was mediated by reconsolidation. Nevertheless, a similar result was observed in the contextual fear conditioning, which has well stablished reconsolidation parameters. Animals exposed to a reactivation session and treated with the amnesic propranolol (noradrenergic β-blocker) expressed impaired memory 24h later. Association with novelty, though, was able to rescue memory from the effect of propranolol and normal retention was observed one day later. This result suggests memory reconsolidation was supported by an association phenomenon, what would indicate underlying STC mechanisms. However, considering the arousal component of the novel experience used, and more importantly, the absence of further evidence, it remains possible that novelty supported reconsolidation by up-regulating neurotransmission in the amygdala, stress hormones, and other systemic processes that do not necessarily involve a process of synaptic tagging and capture (Gold and McGaugh, 1975; Cahill and McGaugh, 1998; De Oliveira Alvares et al., 2010; Wang et al., 2010). Therefore, the involvement of STC mechanisms in the process of memory destabilization and reconsolidation still requires further demonstration and systematically validation for more comprehensive evidence.

Chapter V

CONCLUSIONS

In this thesis we have presented three main studies, two regarding the transition of reconsolidation to extinction of contextual fear memories (Chapters II and III), and one that approached the mechanisms of reconsolidation under the synaptic tagging and capture perspective (Chapter IV).

On the transition of reconsolidation to extinction, it has been described recently a null-point period during which memory was not sensitive to amnesic manipulations (Flavell and Lee, 2013; Merlo *et al.*, 2014; Alfei *et al.*, 2015). However, whether this represented a genuine null-point, during which neither reconsolidation or extinction were engaged, or if it actually represented a period where both processes could be found in the variability of a population, remained unclear (Cassini *et al.*, 2017). Moreover, relatively few studies have previously addressed the intermediary reactivation conditions that lead to either reconsolidation or extinction (Table 2.1), and more evidence has still been required for the validation of the null-point as a conspicuous phenomenon in memory post-reactivation processing. Therefore, our main objective here was to expand our knowledge on the null-point phenomenon and its associated properties.

For that, we submitted rats to a contextual fear conditioning paradigm followed two days later by reactivation sessions of several durations (3, 5, 10, 20 and 30 min). To test whether the diverse conditions have initiated processes of reconsolidation or extinction, during which memory is temporally unstable and thereby susceptible to modifications, reactivation sessions were associated with the amnesic drug MK-801 (Flavell and Lee, 2013; Exton-McGuinness *et al.*, 2014; Zhang, Li and Wang, 2017). First, we opted for a pre-reactivation administration of the drug (Chapter II) and later for post-reactivation injections (Chapter III).

Despite offering interesting insights, the pre-reactivation administration of MK-801 did not allow for clear validation of the parameters required to trigger either reconsolidation nor extinction in the contextual fear conditioning, making it not possible to properly study the intermediary conditions we aimed for on chapter II. Therefore, we transitioned to post-reactivation drug injections that are presented on chapter III. There we observed a significant effect of MK-801 with either the short (3 min) and the long (30 min) reactivation conditions, but no effect when the reactivation conditions were of intermediate duration (5, 10 and 20 min). Additionally, there were no indications of a continuous distribution in the population of both extinction and reconsolidation under intermediary conditions when behaviour was extensively evaluated and replicated. These results, together with later findings in the literature (Merlo, Milton and Everitt, 2018), support the existence of a null-point period in memory processing during which memory reactivation does not result in either reconsolidation nor extinction.

On chapter IV, we used the conditions shown on chapter III to induce memory destabilization (3-min reactivation) and impair reconsolidation (post-reactivation MK-801), to investigate the mechanisms involved under the perspective of the synaptic tagging and capture (STC) hypothesis (Frey and Morris, 1997; Barco, Lopez de Armentia and Alarcon, 2008; Redondo and Morris, 2011). Both consolidation and reconsolidation of a memory are known to rely, at the cellular level, on synaptic plasticity and protein synthesis, but how new synthetized proteins on a neuron soma come to support plasticity specifically and particularly on synapses actively involved with the memory`s encoding, on an infinitive of synaptic connections hold by a neuron, has been until recently not systematically explored. On the past ten-eleven years though, cumulative evidence has been indicating the specificity on synaptic plasticity that supports the establishment of a new memory may rely on mechanisms similar to the ones proposed by the STC concept (Moncada and Viola, 2007; Ballarini *et al.*, 2009; Wang, Redondo

and Morris, 2010; Moncada *et al.*, 2011; Cassini *et al.*, 2013). Although more direct evidence is still required for an effective demonstration of STC mechanisms in the process of learning and memory, the correlates are well promising, and evidence is growing (Table 4.1). Until very recently, however, the concept has yet not been applied to the processes of memory labilization followed by reconsolidation, which also requires protein synthesis and synaptic plasticity (Tronson and Taylor, 2007; Dudai, Karni and Born, 2015; Sweatt, 2016). Here, therefore, we aimed to investigate the possibility of STC mechanism been involved not only with memory consolidation, but also the process of destabilization and reconsolidation of previously acquired memories.

For that, we submitted rats to a brief reactivation session followed my amnesic treatment (MK-801), as described on chapter III, preceded one hour before by a new strong event (open field exploration) (Vianna et al., 2000; Martínez et al., 2012). The STC hypothesis predicts under these conditions an association phenomenon, where the novel strong experience would support the process of reconsolidation and prevent the disruption caused by MK-801 on the contextual fear memory (Fig. 4.1) (Frey and Morris, 1997; Redondo and Morris, 2011). Here we found that indeed no effect of MK-801 was observed under these conditions, which, together with the more recent publication from Wang (2018), offers an initial but interesting indication for the involvement of STC mechanisms in the process of memory destabilization/reconsolidation.

Although these findings require further investigation, systematically replication and more direct observations, the application of the STC concept on memory destabilization and reconsolidation brings a different mechanistic perspective to the process that may result in important and relevant advance on the field and clinical applications (Beckers and Kindt, 2017; Dunbar and Taylor, 2017; Elsey and Kindt, 2017). It may bring a better understanding on how

memory destabilization is initiated cellularly, with potential new targets and possibilities for the treatment of pathological memories that are resistant to labilization and further interference, such as posttraumatic stress disorder (Schwabe, Nader and Pruessner, 2014) and drug addiction (Rich and Torregrossa, 2018). Moreover, the association phenomenon implicated may explain occasional failure of reconsolidation-interfering treatments on the literature and should be considered on future approaches to reconsolidation based-therapies, having in mind events occurring either before or after may have an important influence on the expected outcome of treatment (Forcato *et al.*, 2009; Sevenster, Beckers and Kindt, 2012; Hardwicke, Taqi and Shanks, 2016). More interestingly, the STC may bring an important contribution in the understanding of the boundaries conditions coordinating memory reconsolidation and extinction, and the null-point period. The properties associated with synaptic tagging, as its time limited availability and stimulation-induced activity (Yao *et al.*, 2008; Lu *et al.*, 2011; Sajikumar and Korte, 2011), may explain the cessation of memory engagement into reconsolidation that seems to be characteristic in the null-point period (Cassini *et al.*, 2017), among other associated properties yet to be observed and discovered.

Beyond the prime focus of study addressed on this thesis, it is worth noticing a constant and significant observation of memory decay following memory reactivation of any duration across all chapters and is indicated on chapter III to not depend on NMDA activity (Figs. 2.4, 3.4, 4.4). What this decay represents, and the mechanisms involved, remain to be investigated in the future. Nevertheless, it indicates a common behaviour outcome, as memory attenuation, may result from a bigger variability of process and phenomena then currently appreciated, that may be worth of further examination and should be considered on the interpretation of results purely based on behaviour (Kimberlin and Winterstein, 2008).

It is also worth noticing some differences we observed with the pre- and post-reactivation administration of MK-801. With the 3-min condition, we did not observe any effect of MK-801 when injected before reactivation, whereas the same manipulation was shown to result in significant amnesia when administered immediately after reactivation. At first sight it may seem a contradictory result, but, when analysed, it may actually offer further support for a differential effect of MK-801 on destabilization and reconsolidation depending on whether it is administered before or after reactivation. On chapter II, we suggested the absence of effect of pre-react MK-801, rather than indicating the reactivation failed to induce memory reconsolidation, could indicate an effect of MK-801 upon the destabilization of the memory. That is, by preventing memory from entering destabilization, the injection of MK-801 before reactivation would protect memory from further disruption, it would not be sensitive to interference. Accordingly, when MK-801 was inject after reactivation of same duration, we observed a reduction in memory expression, indicating the reactivation was sufficient to trigger a process of destabilization/reconsolidation, and bring memory to a susceptible state. This corroborates with an effect of MK-801 on destabilization when administered before contextual fear reactivation. Now, it also raises the question of why post-reactivation inhibition of NMDA receptors with MK-801 did not also affect destabilization, since other studies with also post-reactivation systemic administration of other drugs, as the LVGCCs antagonist nimodipine (Suzuki *et al.*, 2008) and the nitric oxide synthesis inhibitors ARL and 3-Br 7-NI (Bal *et al.*, 2017), still reported destabilization impairment when associated with amnesic treatment mediated by protein synthesis inhibition. The answer may rely on the different targets involved and differential involvement with destabilization and reconsolidation process. As we discussed before, NMDA receptors seem to be involved early in the process during destabilization (Mamou, Gamache and Nader, 2006; Bustos et al., 2010; Hong et al., 2013; Ortiz et al., 2014;

Espejo et al., 2016) as well as later on during the re-stabilization phase of reconsolidation (Brown et al., 2008; Brabant, Charlier and Tirelli, 2013; Ribeiro et al., 2013; Lee and Flavell, 2014; Heath et al., 2015). LVGCCs and nitric oxide, however, may be more exclusively involved with destabilization (Itzhak, 2008; Suzuki *et al.*, 2008; Kim, Moki and Kida, 2011; Balaban *et al.*, 2014; Bal *et al.*, 2017). On the other hand, protein synthesis is a phenomenon selectively related to reconsolidation that does not seem to be involved with destabilizion (Tronson and Taylor, 2007; Roesler, 2017; Wang *et al.*, 2018), which actually seems to depend on oppose processes as protein degradation (Dong *et al.*, 2008; Kaang, Lee and Kim, 2009; Lee, 2010). Therefore, even if a post-reactivation drug administration has somehow a reduced window of action upon the early phase of post-reactivation memory processing, i.e. destabilization, its combination with a more selective reconsolidation impairment treatment, would allow for the detection of significant effects over destabilization. This could mean MK-801 injected after memory reactivation, despite a preferential action upon reconsolidation, may still have had a partial effect upon destabilization, and prevented a full action of the drug upon reconsolidation. If true, administration of MK-801 concomitantly with anisomycin, for example, could reduce the efficacy of the protein synthesis inhibitor in impairing memory reconsolidation. However, it could also potentiate its effect by further exerting amnesia on the reactivated memory (Ribeiro et al., 2013; Lee and Flavell, 2014; Heath et al., 2015), so, it is somehow difficult to demonstrate whether post-reactivation administration of MK-801 has some partial effect over destabilization. Nevertheless, it is an important point to be considered when using MK-801 and other NMDA-antagonists to target reconsolidation. Although it does not refute the amnesic effects observed on the literature, the absence of effect may require some revision, especially when referring to the null-point of memory which has been mainly observed with NMDA receptor manipulations (Flavell and Lee, 2013; Merlo *et al.*, 2014; Cassini *et al.*,

2017; Merlo, Milton and Everitt, 2018). Therefore, the use of protein synthesis inhibitors and more clearly selective manipulation of reconsolidation may be of fundamental importance for the validation of the null-point as a general and genuine phenomenon related to memory reactivation.

In addiction to the 3-min condition, we also observed different results with pre and post MK-801 manipulation in the 5- and 30-min reactivations. On Chapter II, pre-reactivation MK-801 was observed to, surprisingly, result in increased expression during the test session, which first called our attention for the potential of MK-801 action over destabilization. We then proposed a hypothetical process of memory weakening mediated by reconsolidation under the conditions of non-reinforced and relatively-brief memory reactivation, which would have been prevented on MK-801 pre-treated animals by avoiding memory destabilization and further modifications. However, if the 5-min reactivation did start memory`s destabilization/reconsolidation, and if post-reactivation has preferential action over reconsolidation, why we do not observe an effect of MK-801 on Chapter III? The answer may rely again on a hypothetical weakening phenomenon mediated by reconsolidation. If, the 5-min reactivation triggered memory destabilization, which then allowed for the weakening of the memory trace through reconsolidation, the amnesic effect of MK-801 over reconsolidation could have been counterbalanced by an increase on freezing expression paradoxically caused by inhibition of the reconsolidation-mediated weakening process. If this is true, although we clearly observed no effect of MK-801 administered after 5-min reactivations, the absence of effect in this case could represent an inability to detect these effects on behaviour rather than then a genuine null-point effect. We could also extend this observation to the longer reactivation durations of 10- and 20-min, which could also have resulted from a similar process on the post-reactivation MK-801 administrations explored in Chapter III. However, it seems to be rather unlikely, since pre-

reactivation MK-801with 10- and 20-min reactivations, differently from 5-min, did not result in higher freezing expression on test, and no evidence for a reconsolidation mediated weakening effect can be detected on those conditions. Moreover, the evidence observed here for the proposed weakening effect is limited and insufficient, and it still possible the effect we observed with the 5-min pre-react MK-801, to be circumstantial and related to an experimental artefact.

With the 30-min condition we observed no effect of pre-reactivation MK-801, which is discussed on chapter II to result from partial action of the drug upon extinction, rather than a failure of the 30-min reactivation to induce extinction. Accordingly, we observed on chapter III a small, but significant effect of MK-801 over memory extinction, further reinforcing the absence of effect on chapter II may have resulted from a reduced action of the drug over the extinction window when administered 30-min previously an additional 30-min reactivation. Nevertheless, the absence of pre-react effect, and the detection of an effect that was significant, but albeit small with post-react administration, may offer further indication for the requirement of longer reactivation duration to result in robust memory extinction with the experimental parameters used here.

It is interesting to note as well that on chapter III not only one, but three different intermediate reactivation conditions resulted in a memory state that was not sensitive to amnesic treatment interference. Oppositely, only two, extreme conditions, were observed to result in the effective engagement of reconsolidation and extinction in the contextual fear memory. Moreover, variations on reactivation manipulations, as different timing of drug administration (chapter II) and addition of relevant experience close in time to reactivation (chapter II) all resulted in not observable change of behaviour despite amnesic administrations. This might indicate that the conditions under which previous memories enter an unstable state that will be sensitive to

modifications are maybe narrower than usually thought. Consequently, there may be a greater possibility of memory reactivation resulting in a memory state that in practice will not be responsive to manipulations, what should be considered in the development of reactivation-based therapies for related memory pathologies, such as posttraumatic stress disorder and drug addiction, as well as memory strengthening strategies on health and disease. (Ballarini *et al.*, 2013; Fitzgerald, Seemann and Maren, 2014; Schwabe, Nader and Pruessner, 2014; Williams and Harding, 2014; Elsey and Kindt, 2017; Rich and Torregrossa, 2018; Wang, 2018).

Finally, although we have added important contribution in the understanding of memory processing beyond reconsolidation and extinction, our knowledge on the null-point properties and the involvement of STC mechanisms are still narrow and under development. Future studies applying diverse methodological approaches to the investigation of intermediate reactivation conditions under different levels of perspective, from molecular biology, to cellular electrophysiology, brain systems and behaviour science, shall be of great value for a more comprehensive understating of the phenomena we report.

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