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Synaptic competition in the amygdala: Heterosynaptic plasticity between thalamic and cortical projections to the lateral amygdala

Ana Margarida Cardoso da Silva Drumond

Orientador: Prof. Doutora Rosalina Maria Regada Carvalho Fonseca de Alvarez

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Dissertação especialmente elaborada para obtenção do grau de
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ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ARC	Activity-regulated cytoskeleton-associated protein
BLA	Basolateral amygdala
Ca^{2+}	Calcium ion
CaMKII	Calcium/ calmodulin dependent kinase II
CB1	Cannabinoid 1
CB1R	Cannabinoid 1 receptors
CeA	Central nucleus of the amygdala
CS	Conditioned-stimulus
CS^{+}	Conditioned-stimulus paired to the US
CS^{-}	Conditioned-stimulus unpaired to the US
CREB	cAMP response element-binding protein
DGAV	*Direção-Geral de Alimentação e Veterinária*
DMSO	Dimethylsulfoxide
eCB	Endocannabinoid

eCBs	Endocannabinoids
EPSPs	Excitatory-postsynaptic potentials
Erg-1	Early Growth Response 1
FAAH	Fatty acid amide hydrolase
GABA	Gamma-Amino Butyric Acid
$G_{i/o}$	G inhibitory/ other
GluR	Glutamate receptor
GTP	Guanosine triphosphate
IEG	Immediate-early genes
I/O	Input/ output
KA	Kainate
LA	Lateral amygdala
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptors
MGm	Medial division of medial geniculate nucleus
MGv	Ventral division of medial geniculate nucleus

mTOR	Mechanistic target of rapamycin
NMDA	N-methyl-D-aspartate
PKA	Protein kinase A
PPF	Paired-pulse facilitation
PRPs	Plasticity-related proteins
RNA	Ribonucleic acid polymerase
STC	Synaptic tag and capture
STM	Short-term memory
$\mathbf{T_{final}}$	Time final
$\mathbf{T_{initial}}$	Time initial
US	Unconditioned stimulus
VGCC	Voltage-gated calcium channels

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RESUMO

Aprender é a principal propriedade do cérebro através da qual, com base na experiência, os animais se adaptam às características do ambiente envolvente. Embora cada evento de aprendizagem possa ser considerado como um novo processo de formação de memórias, existem evidências de que as memórias são formadas sobre uma rede de informações pré-existentes.

É comumente aceite que as memórias evoluem ao longo do tempo, sendo que, logo após a aprendizagem, as memórias são instáveis e suscetíveis de serem modificadas ou perdidas por interferência de eventos concorrentes. Estas são chamadas memórias de curta duração (*short-term memories* – STM), em que através de um processo dependente da síntese de novas proteínas denominado por consolidação, são estabilizadas em memórias de longa duração (*long-term memories* – LTM). A reativação das LTM torna-as instáveis pelo que para se tornarem novamente estáveis, necessitam de ser reconsolidadas, através de um processo denominado por reconsolidação. Após a reativação de uma memória existe um processo alternativo, definido como extinção, que resulta de uma exposição mais prolongada e diminui a expressão da memória inicial. Assim, após a reativação de uma memória adquirida, a sua expressão pode ser mantida ou aumentada por reconsolidação, ou reduzida por extinção. Neste contexto, a reativação de memórias previamente consolidadas, após a aquisição de novas memórias, pode resultar na manutenção de uma LTM por mecanismos de cooperação, ou no seu enfraquecimento por competição.

Os modelos celulares mais aceites que explicam a formação de memórias traduzem alterações na força das sinapses e consistem em modelos de plasticidade sináptica. Nestes, formas duradouras de plasticidade de sináptica, como a potenciação de longa duração (*long-term potentiation* – LTP), também são caracterizadas como tendo diferentes etapas. Mais concretamente, formas transientes de LTP podem serem relacionadas com as STM, e formas persistentes de plasticidade (que são mais estáveis e dependem de síntese proteica) podem ser relacionadas com as LTM. Esta visão de que formas persistentes de LTP dependem de síntese proteica, e que a indução de LTP só ocorre em sinapses previamente ativadas, sugere que as sinapses são marcadas por um sinal molecular local (*tag*), com o objetivo das proteínas associadas à plasticidade (*plasticity-related proteins* – PRPs) serem alocadas. Assim, dado que a plasticidade sináptica

depende da alocação de PRPs e que as sinapses partilham as PRPs disponíveis, para que formas persistentes de plasticidade sejam induzidas, as sinapses terão de cooperar ou competir entre si.

Com o intuito de compreender melhor de que forma estes mecanismos de cooperação e de competição sináptica interferem no processo de formação de memórias, o nosso grupo decidiu estudar estes mecanismos de plasticidade sináptica na amígdala, mais concretamente na amígdala lateral (*lateral amygdala* – LA). A LA e as suas vias aferentes talâmicas e corticais formam um circuito necessário para a formação de memórias condicionadas pelo medo (*fear conditioning memories*). Dado que este circuito se encontra muito bem descrito sob o ponto de vista anatómico e comportamental, é um modelo que permite ligar a fisiologia celular com o comportamento.

O principal modelo celular subjacente ao paradigma de condicionamento por medo auditivo (*auditory fear conditioning*) consiste numa forma de LTP induzida pela associação entre as projeções talâmicas auditivas e corticais auditivas (estímulo condicionado/ *conditioned-stimulus* – CS) e o estímulo nociceptivo (estímulo não condicionado/ *unconditioned-stimulus* – US). Estudos recentes do nosso grupo demonstraram que as sinapses corticais e talâmicas cooperam, resultando na manutenção de formas transientes de LTP por partilha de PRPs entre os dois grupos de sinapses ativadas, e no reforço de ambos os *inputs* de um modo associativo. Este mecanismo de cooperação demonstra ser bidirecional e ocorrer dentro de uma janela temporal prolongada. Contudo, esta partilha revela ser assimétrica dado o facto da capacidade das sinapses talâmicas capturarem PRPs decair muito mais rapidamente comparativamente com as sinapses corticais. Além disso, demonstrou-se que a janela temporal da cooperação talâmica é limitada pela ativação do receptor *cannabinoid 1* (CB1), em que a inibição dos receptores dos endocanabinóides permite estender a janela de cooperação cortico-talâmica.

Com a realização deste trabalho pretendemos abordar, a nível celular, de que forma os mecanismos de cooperação e de competição contribuem para a formação e manutenção de memórias, utilizando como modelo de aprendizagem a formação de memórias de medo associativo (*associative fear memories*). Assim, o primeiro objetivo deste trabalho consistiu em testar se as sinapses talâmicas e corticais interagem por cooperação sináptica, e confirmar que este mecanismo é dependente de síntese proteica. Seguidamente, pretendeu-se testar se estas mesmas sinapses interagem por competição sináptica, determinar quais as regras temporais desta forma de plasticidade e qual o impacto da

modulação da ativação dos recetores CB1. Para testar as hipóteses acima referidas, recorremos à técnica de *patch-clamp* em *current-clamp*, na configuração *whole-cell*, e registámos potenciais excitatórios pós-sinápticos em neurónios piramidais na LA, desencadeados pela estimulação dos inputs talâmicos e corticais.

Numa primeira abordagem, a associação das vias corticais e talâmicas foi testada através da coativação dos inputs talâmicos e corticais, por estimulações fracas (*tetanus* fracos). Verificámos que o LTP das duas vias não se revelou persistente ao longo do registo. De seguida optámos por associar uma estimulação cortical forte (*tetanus* forte) sucedida por uma estimulação talâmica fraca, e desta forma confirmámos a existência de cooperação sináptica. Com recurso a um inibidor da síntese proteica, comprovámos que este mecanismo é dependente de síntese proteica.

Com a estimulação de uma projeção talâmica adicional (por um *tetanus* fraco), foi possível verificar que as sinapses talâmicas e corticais competem quando é gerado um desequilíbrio entre o número de sinapses ativadas e a quantidade de PRPs disponíveis. Demonstrámos que a competição sináptica é modulada pelo tempo, pois o aumento da janela temporal (30-min) da segunda estimulação talâmica diminuiu a competição sináptica, e que esta está relacionada com a disponibilidade reduzida de PRPs.

À semelhança da cooperação, a ativação dos receptores CB1 também modula a competição sináptica. Relativamente a este último aspeto, a inibição dos receptores CB1 leva a um aumento da competição; e um aumento da ativação dos receptores CB1, por aumento da disponibilidade de endocanabinóides, resulta numa diminuição da competição. O facto dos receptores CB1 modularem a força do *tag* das sinapses talâmicas poderá explicar estes resultados.

Verificámos ainda que tanto a competição como a cooperação resultam de um balanço entre a excitação e a inibição, uma vez que inibindo os recetores $GABA_A$ (*Gamma-Amino Butyric Acid*) a cooperação é facilitada.

Assim, os nossos resultados demonstram que os *inputs* corticais e talâmicos para a LA podem interagir entre si dentro de determinadas janelas temporais, competindo quando a disponibilidade de PRPs e o número de sinapses se encontra desequilibrada.

Um aspeto interessante é a possível relação com a aprendizagem discriminativa, quando um animal aprende a discriminar um CS^+/US de uma associação CS^-/US. Nesta situação, os neurónios piramidais da LA aumentam as suas respostas ao CS^+ e mostram

uma diminuição paralela no CS^-. Uma das hipóteses baseia-se no facto desta diminuição da CS-resposta ser resultante de mecanismos de competição sináptica que ocorrem durante a aprendizagem. Em concordância com este mecanismo, o aumento da disponibilidade de PRPs diminui a aprendizagem discriminativa.

Estas observações geram um grande impacto na estrutura conceptual da aprendizagem de medo associativa (*associative fear learning*), uma vez que fornecem um mecanismo celular para a integração contínua da informação nas sinapses da LA. Além disso, ao trabalhar numa área do cérebro bem caracterizada sob o ponto de vista comportamental, este projeto oferece a possibilidade de integrar informações de diferentes níveis de investigação, conduzindo a uma visão unificadora da formação de memórias.

Palavras-chave: Amígdala lateral, cooperação sináptica, competição sináptica, receptores CB1

ABSTRACT

Learning is the main property of the brain through which, based on experience, animals learn to adapt to the characteristics of the environment. Although each learning event can be considered as a new memory formation process, there is evidence that memories are formed over a network of preexisting information. It is commonly accepted that memories evolve over time, and soon after learning, memories are unstable and susceptible to be modified or lost by interference from competing events.

The most accepted cellular models that explain memory formation translate the changes in the strength of the synapses, and consist of models of synaptic plasticity. Long-term potentiation (LTP) requires input-specific allocation of plasticity-related proteins (PRPs) for its maintenance. This view that persistent forms of LTP depend on protein synthesis, as well as the induction of LTP only occurs in previously activated synapses, suggests that these synapses are marked by a local molecular signal (tag), allowing PRPs to be allocated. Thus, since synaptic plasticity depends on the allocation of PRPs, in which synapses share the available PRPs, to induce persistent forms of plasticity synapses will have to cooperate or compete.

In order to better understand how synaptic cooperation and competition are orchestrated as well as their implication in memory formation, we have studied the interaction between the cortical and thalamic afferents to projection neurons of the lateral amygdala (LA). This circuit is known to be involved in the formation of fear conditioning memories. The leading cellular model underlying auditory fear conditioning is a form of Hebbian LTP, induced by the association between the auditory thalamic and auditory cortex projections (conditioned-stimulus – CS) and the nociceptive input (unconditioned-stimulus – US). Recent studies from our group have demonstrated that cortical and thalamic synapses cooperate, resulting in the maintenance of transient forms of LTP by sharing PRPs between these groups of activated synapses. In addition, the temporal window for thalamic cooperation is limited by the activation of the cannabinoid 1 (CB1) receptor.

The goal of this work was to assess, at the cellular level, how the mechanisms of cooperation and competition contribute to the formation and maintenance of memories, based on associative fear learning. Thus, the first objective of this work was to test whether thalamic and cortical synapses interact through synaptic cooperation, and confirm that this mechanism depends on protein synthesis. Next, we wanted to test if these synapses interact

by synaptic competition, uncover what are the temporal rules of this form of plasticity and assess the impact of eCBs receptors activation. We recorded excitatory post-synaptic potentials in pyramidal neurons in the LA, evoked by stimulation of thalamic and cortical inputs. We found that cortical and thalamic synapses can cooperate by sharing PRPs, resulting in the re-enforcement of both inputs. Nevertheless, thalamic and cortical synapses also compete. The stimulation of an additional thalamic projection leads to an unbalance between the number of activated synapses and PRPs availability, resulting in competition. Synaptic competition is modulated by time, whereas extending the time window decreases synaptic competition, and depends on the reduced availability of PRPs. Interestingly, we have further found that both competition and cooperation result from a balance between excitation and inhibition since $GABA_A$ receptors blockage enhances cooperation. Activation of the endocannabinoid receptor CB1 (CB1R) also modulates synaptic competition – increased activation of CB1R decreases competition and CB1R blockage enhances competition.

Our results show that cortical and thalamic inputs to the LA can interact with each other within large time windows, competing when the availability of PRPs and the number of activated synapses is unbalanced. This observation has a profound impact on the conceptual framework of associative fear learning, as it provides a cellular mechanism for continuous integration of information at amygdala synapses.

Keywords: Lateral amygdala, synaptic cooperation, synaptic competition, CB1 receptors

CHAPTER 1

INTRODUCTION

MEMORY DYNAMICS

The brain is an organ with a highly capacity to adapt, managing to convert sensory information into neuronal function changes. Learning is the core property of the brain by which, based on experience, allows animals to adjust to the environment, contributing to adapt their behaviour (Kandel, 2001). It is commonly acknowledged that learning produces changes in neuronal activity pattern within the brain and that different brain networks mediate distinct types of learning, and thus different memories (Dayan and Abbott, 2001; Pavlov, 1927; Redondo and Morris, 2011). Although learning and memory are integrated terms, distinct neural phenomena differ each other – learning is the process of information acquisition that modifies a certain behaviour and occurs slowly, while memory is the ability to remember past experiences and occurs rapidly (Kandel, 2001; Packard and Goodman, 2013). Curiously, learning can be considered as new memory formation process, in which molecular and cellular events lead to stable changes in neuronal activity (Nader et al., 2000b).

Despite the earlier knowledge that memories are permanently storage (Glickman, 1961), the majority of studies on memory suggest that memory evolves over time, wherein new memories are modulated by preceding and upcoming events (Nader, 2015; Tronson et al., 2006). It is now consensual that the mechanisms involved in initial memory acquisition (encoding and storage), consolidation, reconsolidation and extinction reflect modifications in dynamic interactions and synaptic efficacy between distinct neuronal networks (Lee, 2013; Tse et al., 2007).

Most studies on memory formation argue that memories can be grouped accordingly to its duration, into short-term memories (STM) and long-term memories (LTM) (Squire, 1986; Tse et al., 2007). Short-term and long-term memories show distinct biological states. STM and LTM appear to have different mechanisms of retention, wherein STM reflect transitory neural changes and LTM generate changes in neural pathways capable of storing information that can be recalled long after (Alberini, 2009). According to these assumptions, memory formation and maintenance have been proposed as a model in stages or steps [**Figure 1**] (Drumond et al., 2016). Soon after learning, memories are transient and unstable, being expressed as STM (Alberini, 2011; McGaugh, 2000). Through a process called consolidation, these transient traces become less labile and more resistant to perturbation,

and are expressed as LTM (McKenzie and Eichenbaum, 2011). The duration of memory consolidation, which differs among species and behavioural paradigms, is defined by dependence on protein synthesis (Alberini, 2011), wherein a STM, in contrast to a LTM, is not dependent on protein translation (Gold, 2009). During the early phase of consolidation, memory formation can be disrupted or prevented by various interferences, including inhibition of transcription factors, blockage of certain molecular pathways, additional learning, among other types of chemical and physical disruptions (Alberini, 2009). Interestingly, if either protein or mRNA synthesis is blocked before or immediately after training, LTM is disrupted (Davis and Squire, 1984). Another interesting aspect is that memory reactivation turns LTM, i.e. previously consolidated memories, into active memories, again vulnerable to disruption (Bouton, 1993). Therefore, if retrieved, memories can either be lost or reconsolidated as stable traces. This process of reconsolidation is also dependent on gene transcription and protein synthesis (Nader, 2015).

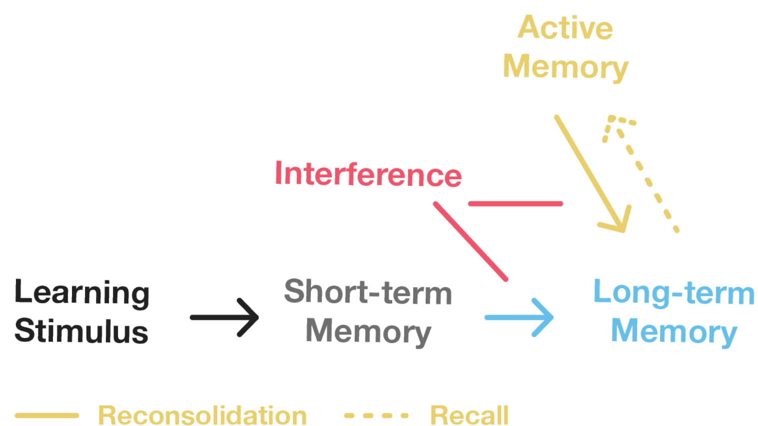


Figure 1: Mechanism of memory formation and maintenance. Learning results in the formation of STM. Through a process of memory consolidation STM can be converted into LTM. Conflicting information or protein synthesis inhibition can interfere with memories not yet consolidated. LTM when reactivated turn into active memories, again susceptible to interference. Active memories become again LTM through a process of reconsolidation. LTM maintenance and updated memories require memory consolidation and reconsolidation.

Although the mechanism of memory formation is considered as an evolution of these memories through various phases or stages, substantial evidence suggests that new memories are formed in an interleaved manner upon a large network of pre-existing information (Robertson, 2012). Thus, this linear model of consolidation and reconsolidation

does not capture the integrative evolution of memory (Robertson, 2012). Since memory reactivation renders pre-acquired memories again labile to disruption, in which a second wave of gene transcription and protein synthesis is triggered (Sara, 2000), it is plausible that new memory formation lead to the maintenance or to disruption of a previously consolidated memory (Lee et al., 2008). In other words, two memories can either re-enforce each other in a cooperative fashion or can compete leading to their loss. Therefore, memory maintenance after acquisition is rather an active process, allowing for memory updating.

Similarly to consolidation and reconsolidation, there is an alternative process, defined as extinction, that is also dependent on *de novo* protein synthesis, despite having distinct biochemical and temporal mechanisms (Suzuki, 2004). However, and contrary to reconsolidation, after memory retrieval extinction decreases the expression of the initially acquired memory, where a new association is stablished, which inhibits the expression of the previous one (Myers and Davis, 2002). Thus, after memory retrieval, the expression of a previous acquired memory can either be maintained or increased, through reconsolidation, or reduced by extinction. The duration of the exposure to retrieval stimuli determines whether reconsolidation or extinction is triggered, in which the latter requires prolonged exposure (Suzuki, 2004). Assuming that acquisition of memories leads to the reactivation of previously consolidated memories, then this could lead to the maintenance of a LTM, in a cooperative manner (if reconsolidation is triggered), or to the weakening of a LTM, in a competitive manner (if extinction is triggered) (Drumond et al., 2016; Suzuki, 2004).

Together, these observations suggest that learning is highly influenced by prior and future experiences occurring in temporal proximity, in which previous consolidated memories, when reactivated during new learning, can be updated or lost (Debiec et al., 2013; McKenzie and Eichenbaum, 2011). Hence, given that memory cooperation and competition appear to have an important role on memory dynamics, the rules governing these mechanisms must be addressed and better understood. Linking the molecular and cellular mechanisms of synaptic plasticity with behaviour is likely the most suitable approach.

CELLULAR MODELS OF MEMORY FORMATION AND LEARNING

Memory storage is believed to be heavily based on changes in synapses, specialized connections between the nerve cells within the nervous system (Martin and Morris, 2002). These changes in synaptic connections are broadly referred to as synaptic plasticity, and they represent one of multiple neuronal plasticity processes (Giese and Mizuno, 2013). Thus, the most recognized model for memory acquisition is based on activity-dependent modifications in synaptic efficacy between neurons of a certain neuronal network, in which neurons that fire in a coordinate fashion are likely to have their connections strengthened (Tonegawa et al., 2015).

The large body of work on synaptic and molecular consolidation has demonstrated that long-lasting phases of memory depend on regulated protein expression (Rosenberg et al., 2014). This sensitivity to manipulations that inhibit protein synthesis has significant parallels within the most accepted cellular models of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD). In addition to the dependence of protein synthesis (Rosenblum et al., 2000), LTP show another similar features with memory formation and maintenance (Malenka and Nicoll, 1999). LTP can also be characterized as having different stages. Upon induction, LTP can be expressed as a transient form of LTP or a persistent/ maintained form of LTP, wherein the first is related to STM, and the second can be related to LTM (Govindarajan et al., 2006; Malenka and Nicoll, 1999). These persistent forms of synaptic plasticity depend on gene transcription and *de novo* synthesis of plasticity-related proteins (PRPs) [**Figure 2**]. These PRPs, which include Homer1a, activity-regulated cytoskeleton-associated protein (ARC) and AMPAR (α-amino-3-hydroxyl-5- methyl-4-isoxazole-propionate receptor) subunit of glutamate receptors (GluR) (Lanahan and Worley, 1998; Miyashita et al., 2008), are then allocated to activated synapses (Sajikumar et al., 2007). Thus, a transient LTP, upon induction, can be stabilized into a maintained form of LTP by allocation of PRPs.

This view that maintained forms of LTP are dependent on gene products translated into PRPs, adding that LTP induction is input-specific (i.e. takes place only at synapses that were activated during the induction) (Bliss and Collingridge, 1993), suggests that synapses are "tagged" so that these plasticity factors can be transported (Frey and Morris, 1997).

The model referred to as the synaptic-tagging and capture (STC) proposes that activated synapses become transiently tagged so that PRPs can be captured, allowing conversion of a transient form of plasticity into a persistent form of LTP (Redondo and Morris, 2011; Reymann and Frey, 2007). Interestingly, this working model demonstrated that the induction of a long-lasting form of LTP in one set of synapses is able to stabilize a transient form of LTP induced in a second independent set of synapses (Frey and Morris, 1997). Consistent with this, the maintenance of LTP can be independent of the neuronal activity occurring during the induction of LTP, yet depending on future and past activity (Redondo and Morris, 2011). This theory has emerged from reports showing that heterosynaptic and homosynaptic forms of synaptic activity modulate the maintenance of persistent and protein synthesis-dependent forms of plasticity (Fonseca et al., 2006).

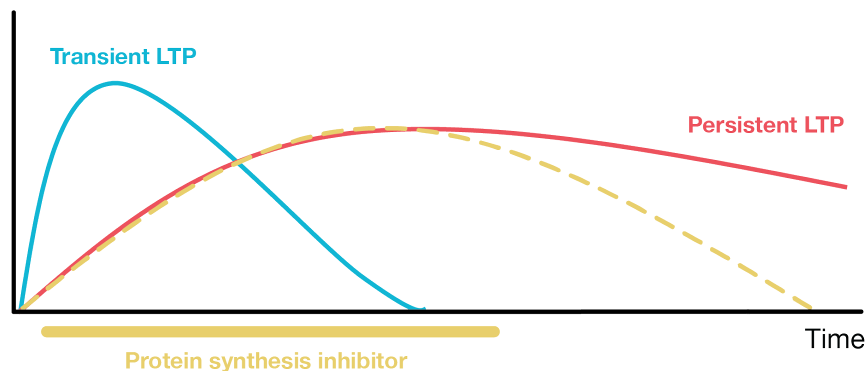


Figure 2: Schematic representation of transient forms of LTP and persistent forms of LTP. Protein synthesis inhibition blocks the induction of persistent forms of LTP.

Protein synthesis, synaptic tagging and synaptic capture of PRPs are distinct processes but essential mechanisms for the stabilization of transient forms of LTP. Remarkably, by cooperative sharing of PRPs, synthesised upon the induction of maintained forms of LTP, a transient form of LTP in another set of tagged synapses can be stabilized. (Redondo and Morris, 2011). In accordance with these previous studies, the stabilization of a transient form of LTP is blocked when protein synthesis inhibitors are applied during the induction of long-lasting forms of LTP (Fonseca, 2013). This suggests that cooperation is achieved by an interaction between the activity-dependent input-specific "synaptic tags" and the capture of PRPs.

STC model was the first demonstration that synapses indeed cooperate by sharing

PRPs. However, there are several aspects and constraints around this model that should be considered. On the one hand, some studies found that the ability to induce synaptic cooperation is dependent on close proximity between synapses (Govindarajan et al., 2011), on the other hand the spatial capacity of tagged synapses to capture PRPs is modulated by the strength of the input stimulation (Alarcon et al., 2006). This means that PRPs are distributed along pools of activated synapses that are spatially close, and only if the tag is strong enough to capture PRPs.

Although the long-lasting maintenance of LTP and the setting of the “synaptic tag” are two independent processes that can occur separately in time (Fonseca, 2012; Sajikumar et al., 2007), maintained forms of LTP require high levels of PRPs available to be allocated at tagged synapses. Thus, if the pool of activated synapses to which PRPs are allocated is increased or the amount of PRPs is reduced, synapses tend to compete for the allocation of these factors, engaging in synaptic competition (Fonseca et al., 2004). Consistent with this, by increasing the availability of PRPs the expression of persistent forms of LTP is also increased (Barco et al., 2002). Therefore, it is plausible to conclude that synapses can either cooperate or compete, depending on the number of activated synapses that can capture PRPs and on the availability of PRPs, resulting in LTP maintenance or disruption respectively.

It seems relevant to address the rules by which synaptic cooperation and competition are orchestrated, as well as their effect on neuronal network activity, as it allows to predict their implications in memory formation processes. Namely, memory maintenance through cooperation is dependent on spatial distance of synapses, sharing of PRPs (wherein the duration of the tag and PRPs availability are time-restricted) and on the number of coincident tags that capture the available amount of PRPs (Moncada et al., 2015).

FEAR LEARNING AS A MODEL TO STUDY COOPERATION AND COMPETITION

The classical Pavlovian fear conditioning is a very attractive and useful behavioural model for investigating the neurobiology of memory formation and maintenance (Pavlov, 1927). This model has proven particularly attractive for many reasons: this type of conditioning occurs widely in the animal kingdom; the training paradigm results in associative learning, which is long-lasting and easily acquired; it allows one to control the induction, expression and extinction of the memory; it involves well-defined stimuli and results in precise responses that can be reliably measured (Ledoux, 2000; Pape and Pare, 2010).

The fear conditioning is a form of associative learning in which subjects begin to express fear responses to a neutral conditioned stimulus (CS), a sensorial stimulus such as a tone/ light or context, that is paired with an aversive unconditioned stimulus (US), such as an electric shock (a nociceptive stimulus). As a result of this pairing, the re-exposure to the CS starts to elicit behavioural and visceral responses that are expressed in the presence of danger, e.g. freezing (Bolles and Fanselow, 1980). This conditioned response depends on the acquisition of an associative memory between the US and the CS that needs to be consolidated to persist (Blair et al., 2001).

A brain region called amygdala is presumed to be the site of association between the CS and the US (Campeau and Davis, 1995; Ledoux, 2014, 2000) and much of the evidence has come from studies of auditory fear conditioning, in which a neutral auditory tone represents the CS. The amygdala is located in the anterior portion of the temporal lobe (part of the limbic system), and is comprised of cortex-like structures and a dozen or so nuclei, which only some components are involved in the acquisition and extinction of conditioned fear responses – the basolateral complex (lateral amygdala (LA), basolateral amygdala (BLA) and basomedial nuclei) and the central nucleus (CeA) (Maren, 2005; Phelps and LeDoux, 2005).

Considerable evidence suggests that the lateral nucleus of the amygdala (LA) is one of the critical sites where the auditory (CS) and somatosensory inputs (US) association occurs. Thus, this means that LA is responsible for the storage of this associative memory

and displays the earliest response to auditory stimuli (Blair et al., 2001; Bordi and LeDoux, 1992; Pitkänen et al., 1997). This is also consistent with work showing that damage to LA prevent the conditioning of fear responses to an auditory CS (LeDoux et al., 1990).

The LA receives direct auditory sensory inputs from the thalamus and cortex, and serves as the sensory interface of the amygdala (Blair et al., 2005; Doron and Ledoux, 1999). The auditory CS information reaches the LA either indirectly via auditory cortex, comprising both the nonlemniscal and lemniscal pathways, or directly from the nonlemniscal auditory thalamus (Boatman and Kim, 2006; Romanski and LeDoux, 1993). Neurons from the nonlemniscal pathway, project from the medial division of medial geniculate nucleus (MGm) mainly into pyramidal cells of the LA, and respond to multiple sensorial stimuli, including somatosensory stimuli and to sound. Whereas in the lemniscal pathway, neurons from the ventral division of medial geniculate nucleus (MGv) of the auditory thalamus are much more accurate, projecting into auditory cortex (A1) and then into LA, and are tonotopically organized (respond according to the frequency of the sound) (Antunes and Moita, 2010; Nader et al., 2000a) [**Figure 3**]. As for the information of the shock (US), it reaches directly the LA, via somatosensory projections (thalamic-independent and thalamic-dependent projections) (Lanuza et al., 2004).

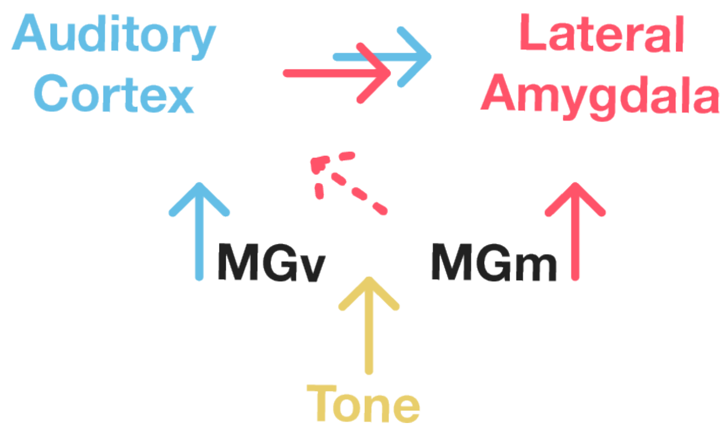


Figure 3: Schematic representation of the inputs into the LA. The tone information is received by the thalamic nuclei MGm and MGv. Then, MGm neurons project strongly to the LA and weakly to the auditory cortex, while MGv neurons project strongly to the auditory cortex.

Assuming the previously stated, since the MGm neurons respond to multiplicity of stimuli (i.e. sound and somatosensory stimuli), the thalamic projections to the LA provide a

less accurate, yet rapid, relay of the auditory information. While the cortical projections, which respond according to the frequency of the sound, are much more accurate and increase the specificity of this pathway (the MGv neurons and auditory cortex are tonotopically organized) (Antunes and Moita, 2010).

The activation of either thalamic or cortical inputs into the LA seems to be sufficient for fear-conditioning learning (Kwon and Choi, 2009). However, activation of both inputs has been shown to be essential for auditory discriminative learning (Antunes and Moita, 2010). In discriminative forms of fear learning, animals learn to fear a conditioned stimulus paired to the US (CS^+), while repressing the responses to a non-paired stimulus (CS^-). In the case of failing to repress the response to the CS^-, this leads to generalization in which fear responses are elicited also by the CS^- presentation (Antunes and Moita, 2010). One idea is that in order to reduce generalization, downregulation of the thalamic projection by competitive interactions between cortical and thalamic inputs may be essential. As MGm neurons will be active by any sensory stimuli that occurs around the time of the conditioning, forming associative memories with any of these stimuli, their downregulation seems to be essential.

Since the LA circuitry is very well described from the behavioural and anatomical point of view, the study of the interactions between cortical and thalamic inputs into the LA may offer the possibility to better understand and assess the rules underlying memory cooperation and competition (Fonseca, 2013; Maren, 2005).

SYNAPTIC PLASTICITY IN THE LATERAL AMYGDALA

Fear conditioning induces changes in synaptic efficacy at afferent synaptic inputs to the LA. This causal relationship has been earlier demonstrated either by recordings of synaptic responses to afferent stimulations in brain slices *in vitro* (McKernan and Shinnick-Gallagher, 1997; Tsvetkov et al., 2002), as well as by intracellular and extracellular recordings of CS-evoked firing *in vivo* (Repa et al., 2001; Rogan et al., 1997). For instance, following conditioning, LA responses to the CS^+ are greater than those after unpaired presentation of the US and CS, and are opposite to those evoked by a CS^- (Corcoran and Maren, 2004; Goosens et al., 2003). Together these data support the notion that the LA is the site of plasticity of an associative nature, since changes in LA responsiveness mirror plasticity occurring in the cortex or thalamus (Cassell et al., 1986).

Available evidence, from neurophysiological and pharmacological studies, strongly suggests that a form of associative Hebbian LTP occur in LA during fear conditioning (LeDoux et al., 1990; Maren, 2005, 2001; Poremba and Gabriel, 2001). According to Hebb's rule, if the same neurons weakly stimulated by the auditory CS, are strongly stimulated, close in time, by the US, the synapses processing the CS are strengthened. This property is commonly referred to as "associativity", meaning that weakly stimulated synaptic inputs can be strengthened by co-activation with strong inputs.

Although these Hebbian neural changes seem to be critical for the conditioning of fear responses to an auditory CS, which more that 70% of all LA neurons receive information regarding the auditory CS, or the US (Quirk et al., 1995), only a smaller subgroup of these neurons are responsible for encoding the memory (Reijmers et al., 2007). Thus, this implies that there are some mechanisms responsible for the allocation of fear memories to specific neurons in the LA.

1. LTP at cortical and thalamic synaptic inputs to the LA

Cortical and thalamic input fibers converge onto both local interneurons and projection neurons of the LA, wherein they even converge onto the same dendrites (Szinyei

et al., 2000). The current knowledge assumes that LTP induction in thalamic and cortical inputs to LA pyramidal neurons involves calcium-dependent activation of protein kinase A (PKA), mitogen-activated protein kinase（MAPK) and calcium/calmodulin dependent kinase II (CaMKII) (Huang and Kandel, 1998; Schafe et al., 2000). Persistence forms of LTP, however, involve postsynaptic AMPA receptor insertion (Rumpel et al., 2005). Additionally, LTP in both cortical and thalamic inputs also depends on voltage-gated Ca^{2+} channels (VGCC) and *N-methyl-D-aspartate* (NMDA) receptors, which shows identical induction mechanisms. Interestingly, NMDA receptors are predominantly located at presynaptic sites of the cortical inputs, and at postsynaptic sites of the thalamic inputs (Szinyei et al., 2000).

Different forms of LTP can be observed at LA synapses, depending on the degree of postsynaptic depolarization, as well as on the presynaptic activity levels (Fourcaudot et al., 2009; Shin et al., 2010). Upon stimulation of postsynaptic NMDA receptors and/ or VGCC, LTP is mostly homosynaptic at cortical or thalamic inputs, meaning that the firing of the LA pyramidal neuron (postsynaptic neuron) is associated with the firing of the presynaptic neuron, either cortical or thalamic (Bailey et al., 2000). Whereas, through concurrent activation of thalamic inputs and cortical inputs, other form of LTP is heterosynaptic upon stimulation of presynaptic NMDA receptors at cortical inputs (Mahanty and Sah, 1999). This shows that LTP can spread to the heterosynaptic pathway by glutamate “spillover” from the thalamic stimulated synapses (Humeau et al., 2003; Tsvetkov et al., 2004). Thus, although LTP in LA is dependent on postsynaptic NMDA receptors, there are some forms of LTP that depend on presynaptic NMDA receptors.

Despite these previous reports proposing that presynaptic forms of cortical LTP are induced by coincident activation of thalamic and cortical inputs, both presynaptic and postsynaptic mechanisms have been reported (Fonseca, 2013; Huang and Kandel, 1998; Humeau et al., 2003). For instance, upon strong tetanic stimulation, postsynaptic forms of cortical LTP were reported, which induction is dependent on NMDA receptors activation (Huang and Kandel, 1998). In addition, upon weak tetanic stimulation of the thalamic input, LTP depends on NMDA activation and is expressed postsynaptically. Strong tetanic stimulation, on the other hand, is expressed pre and postsynaptically (involves NMDA, VGCC and KA receptors). This suggests that depending on the afferent stimulation protocol, presynaptic and postsynaptic forms of LTP can be induced.

The above mentioned proposes that cortical and thalamic inputs to LA neurons

express different types of plasticity associated with contrasting forms of coincidence detection as well as overlapping.

2. Synaptic cooperation in the LA and role of the eCB system

The maintenance of LTP, although depending on the activity that occurs during the induction of LTP, is especially dependent on future and past neuronal activity (Redondo and Morris, 2011).

It is consensual that LTP induction in thalamic and cortical inputs to LA principal neurons displays input specificity and to persist requires *de novo* protein synthesis (Huang and Kandel, 2007; Schafe and LeDoux, 2000). STC hypothesis argues for a cellular mechanism that enables the association of events that occur separated by large time windows, where activated synapses become transiently tagged allowing the subsequent capture of PRPs (Redondo and Morris, 2011). Associativity implies that cooperativity also exists, wherein the first one implies that LTP can be induced in some set of inputs only when they are stimulated in combination with another set, and the second implies that a minimum number of inputs must be activated in order to induce LTP in those inputs (Johnston and Miao-Sin Wu, 1995). Thus, it becomes relevant to assess whether cooperative associative LTP induction and maintenance, through mechanisms of STC, is observed in LA synapses.

Associated forms of plasticity involving coincident activation of thalamic and cortical inputs have been reported (Humeau et al., 2003). However, a different form of associative plasticity have been recently demonstrated, in which activation of a subsequent heterosynaptic input, is able to reinforce a transient form of homosynaptic LTP (Fonseca, 2013). In other words, cortical and thalamic inputs into the LA engage in synaptic cooperation, in which induction of transient forms of LTP, induced by weak stimulation of cortical or thalamic inputs, can be maintained by the capture of PRPs synthesized after an heterosynaptic strong LTP induction (Fonseca, 2013) [**Figure 4**]. The author showed that this cooperation leads to the re-enforcement of both inputs in an associative manner, even within wide time window, and that cortical-thalamic cooperation is bi-directional but temporally asymmetrical. Specifically, in this report different time windows between cortical strong stimulation and thalamic weak stimulation, as well as between thalamic strong stimulation and cortical weak stimulation were used. The author found that the ability to

capture PRPs by activated thalamic synapses decays much faster (7.5-min) than the ability of cortical synapses (30-min), which suggests a restriction mechanism in thalamic cooperation (Fonseca, 2013). Although these heterosynaptic associative forms of LTP share a common pool of PRPs, this work proposes the existence of distinct time constraints for cooperation due to some synaptic modulators.

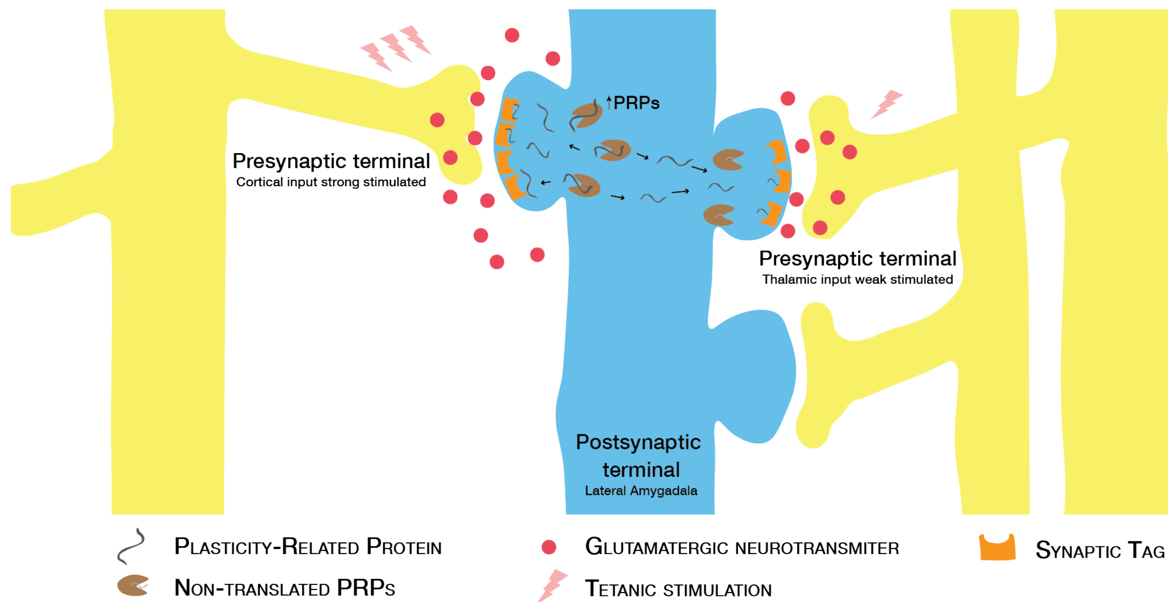


Figure 4: Potential mechanism for cortical-to-thalamic cooperation. Strong tetanic stimulation of the cortical input (glutamatergic neurons) induces a maintained form of LTP and results in the upregulation of PRPs at the postsynaptic terminal. Then, these PRPs are capture at the thalamic (weak stimulated) and cortical tagged synapses.

Interestingly, inhibition of the cannabinoid receptor 1 (CB1R) extends the time window for cortical-to-thalamic cooperation, through the activation of postsynaptic metabotropic glutamate receptors (mGluR) (Fonseca, 2013). CB1R, which are coupled to G_i/G_0 proteins, are expressed very heavily in the presynaptic terminals to the amygdala, particularly in the thalamic inputs (Freund et al., 2003; Katona et al., 2001). When thalamic synapses are highly active, endocannabinoids (eCBs) are synthesised and released from the post-synaptic compartment, reducing the activity of thalamic synapses after binding to the presynaptic receptor. Therefore, the thalamic synaptic cooperation restriction mechanism might be due to the release of eCBs (Azad, 2004; Shin et al., 2010). This is consistent with previous work reporting that upon induction of a postsynaptic thalamic LTP

presynaptic thalamic LTP is suppressed, which in this case, the retrograde signaling of the eCBs via activation of the presynaptic CB1R might be responsible (Fonseca, 2013; Shin et al., 2010). Recent studies report that CB1R activation modulates PRPs synthesis (Busquets-Garcia et al., 2013), thus one can consider that CB1R activation restricts synaptic cooperation by reducing the availability of PRPs. Another interpretation is that activity-dependent release of eCB might be restricting the ability of thalamic synapses to capture PRPs synthetized upon cortical heterosynaptic strong LTP induction (Fonseca, 2013).

Some reports have shown that both cue-fear and generalized responses during fear conditioning are modulated by CB1R activation (Reich et al., 2008). Since CB1R activation might be related with fear generalization, it is plausible to assume that by limiting the time window in which thalamic synapses can cooperate with cortical synapses, this cellular mechanism is limiting incorrect associations and consequently generalization (Fonseca, 2013; Maren, 2005).

3. GABAergic plasticity

The neuronal composition of the LA contains two classes of neurons, wherein the dominant group (80%) consists of glutamatergic neurons with multipolar dendritic trees covered with spines and axons, and the second class (20%) consists of local circuit GABAergic neurons with short axons and spiny dendrites (Faber et al., 2001; Pape and Pare, 2010). Although most research has focused on the role of glutamatergic plasticity and transmission, there is evidence that local inhibitory circuits in the amygdala mediate important aspects of fear conditioning (Ehrlich et al., 2009).

Previous reports showed that GABAergic population have an important role in modulating synaptic plasticity in the LA (Danober and Pape, 1998). This is consistent with works demonstrating that LA projection neurons receive substantial GABAergic feedforward inhibition, which closely controls their activity (Ehrlich et al., 2009; Szinyei et al., 2000), and LA interneurons receive in turn convergent cortical and thalamic afferents (Szinyei et al., 2000).

GABA released from axon terminals can act on two distinct classes of receptors: $GABA_A$ receptors, which are ligand-gated chloride channels; and $GABA_B$ receptors, which are G-protein coupled, inhibitory receptors (Olsen and Sieghart, 2009; Ulrich and Bettler, 2007). Remarkably, in the LA, $GABA_A$ receptors are expressed postsynaptically and control mainly thalamic afferents, and $GABA_B$ are expressed presynapically, controlling cortical afferents (Ehrlich et al., 2009; Szinyei et al., 2007).

GABAergic influences are different under baseline conditions and upon fear conditioning. In baseline conditions, through activation of presynaptic $GABA_B$ receptors at afferent inputs and postsynaptic $GABA_A$ receptors, synaptic plasticity in principal neurons is inhibited. However, in fear conditioning, extracellular GABA concentration decreases, reducing the blockage from presynaptic $GABA_B$, which relieves glutamatergic inputs and facilitates LTP (both heterosynaptic cortical and postsynaptic thalamic types). Therefore, through some of these mechanisms GABAergic regulation of synaptic plasticity appears to be involved in the CS-specificity of the conditioned responses, as well as conditioned fear induction itself.

Plenty work have shown that the induction of LTP in LA principal neurons can suppress inhibition from local interneurons (Li and LeDoux, 1995). Recently it was found that a competitive process between excitation and inhibition determines whether memories become bound or are segregated in the LA (Rashid et al., 2016). This form of competition is likely to be involved in the integration of memories for events occurring shortly in time and in the distinction of events occurring farther apart in time (Rashid et al., 2016). Thus, since synaptic $GABA_A$ receptors mediate phasic (or fast) inhibition in the LA, an essential component of this fear circuit (Sanders and Shekhar, 1991), their action is presumably to restrict synaptic plasticity mechanisms between cortical and thalamic inputs into the LA.

CHAPTER 2

OBJECTIVES

We aimed to better understand how synaptic cooperation and competition are orchestrated as well as their implication in memory formation. Therefore, we have studied the interaction between the cortical and thalamic afferents to projection neurons of the LA. By discriminating the synaptic rules underlying synaptic plasticity in the LA synapses, this provide us with valuable information to better understand the cellular mechanisms underlying associative and discriminative fear learning.

In this context, the specific aims of this work were:

1. Assess whether thalamic and cortical synapses interact by synaptic cooperation;
2. Assess whether synaptic competition occurs between thalamic and cortical inputs activation;
3. Determine the temporal rules and assess the mechanistic constraints underlying synaptic cooperation and competition in LA;
4. Test whether endocannabinoid signaling plays a role in synaptic competition.

CHAPTER 3

METHODS

SLICE PREPARATION

All experiments were performed in coronal brain slices taken from male Sprague-Dawley rats (21–32 days old). The animals were decapitated under isoflurane anesthesia and the brains were quickly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF). All procedures were approved by the Portuguese Veterinary Organization (DGAV).

Coronal brain slices (350 µm thick) containing the lateral amygdala nuclei [**Figure 5**] were prepared using a vibrotome (Leica, VT1200S) in an ice-cold cutting ACSF saturated with 95%O_2/5%CO_2 and containing (in mM) 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 5 $MgCl_2$, 1 $CaCl_2$ and 25 glucose. Slices were maintained in cutting ACSF at 32ºC for at least 1h before being transferred to a recording submersion chamber, and perfused continuously with recording ACSF at 32ºC. The recording ACSF was saturated with 95%O_2/5%CO_2 and contained (in mM) 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_2, 26 $NaHCO_3$, 1.5 $MgCl_2$, 2.8 $CaCl_2$, 25 glucose.

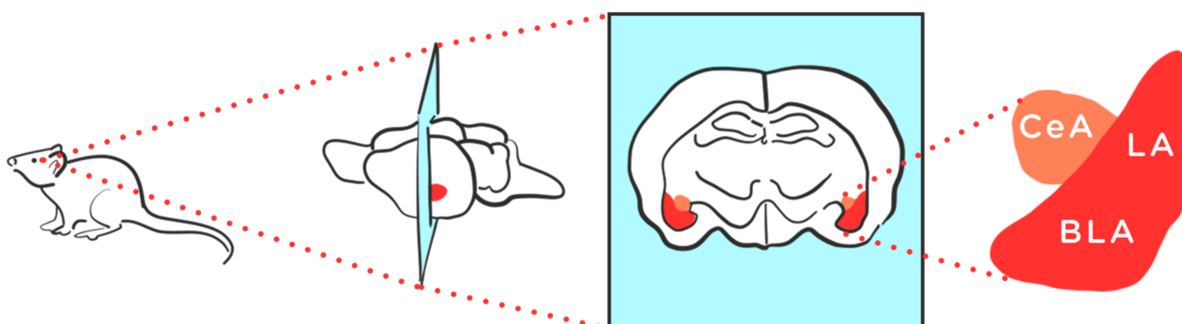


Figure 5: An enlarged image of amygdala nuclear groups. The basolateral complex (LA and BLA) and central nucleus (CeA) are shown next to a coronal section from the brain of a rat.

ELECTROPHYSIOLOGICAL RECORDINGS

Whole-cell current-clamp recordings from pyramidal neurons were obtained with glass electrodes pulled from a 1.5mm outer diameter and 0.86mm inner diameter thin-walled capillary tube (Harvard apparatus, UK) obtaining a resistance of 5–7MΩ when filled with an internal solution containing (in mM): K-gluconate 120, KCl 10, HEPES 15, Mg-ATP 3, Tris-GTP 0.3 Na-phosphocreatine 15 and creatine kinase 20U/ml (adjusted to 7.28-7.32 pH with KOH, 285-305 mOsm).

Putative pyramidal cells from the lateral amygdala were selected by assessing the firing properties in response to steps of current [**Figure 6**]. Only cells that had a resting potential of less than -60 mV without holding current were taken further into the recordings. Neurons were kept at -70 to -75 mV with a holding current below 0.25 nA.

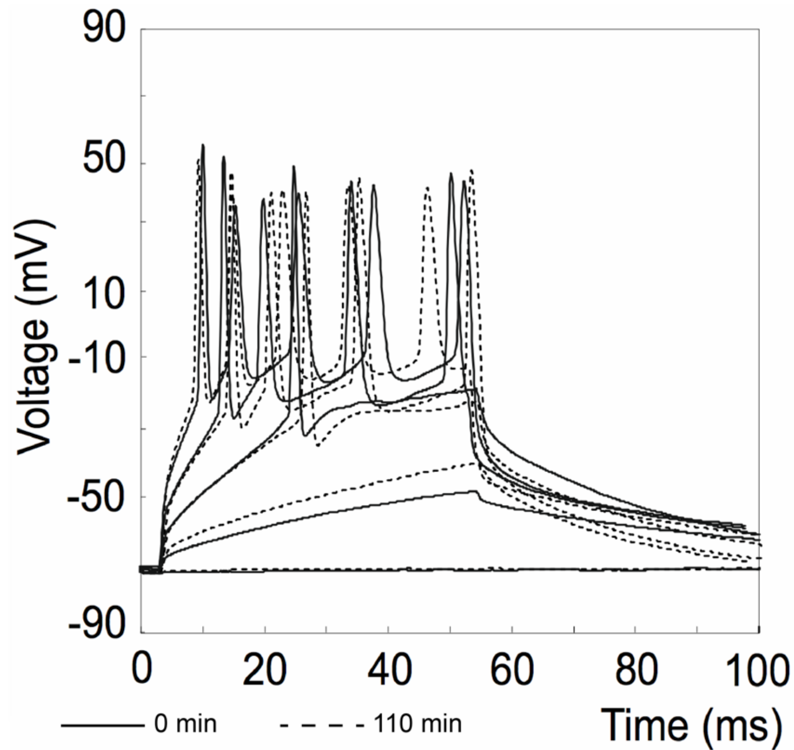


Figure 6: Voltage responses of a LA pyramidal neuron cell in response to steps of depolarizing current injections.

Stimulating electrodes (monopolar tungsten electrodes – Science Products, GmbH, Germany) were placed on afferent fibers from the internal capsule (thalamic inputs) and from the external capsule (cortical input) [**Figure 7**]. Cortical and thalamic inputs were stimulated with 0.2 ms pulses. Pathway independence was checked by applying two pulses with a 50-ms interval to either the cortical or the thalamic input; the absence of crossed pair-pulse facilitation (PPF) was confirmed if no change in the signal of any of the pathways was observed. The test pulse frequency for each individual pathway was 0.033 Hz, except in the experiments in which only two pathways were recorded in which the test pulse frequency of each individual pathway was 0.022Hz. Stimulus intensities were set to evoke 50% of the maximal excitatory postsynaptic potential (EPSP) amplitude and LTP was induced after

recording a stable baseline of EPSPs for 20-min. EPSP were continuously recorded for at least 110-min.

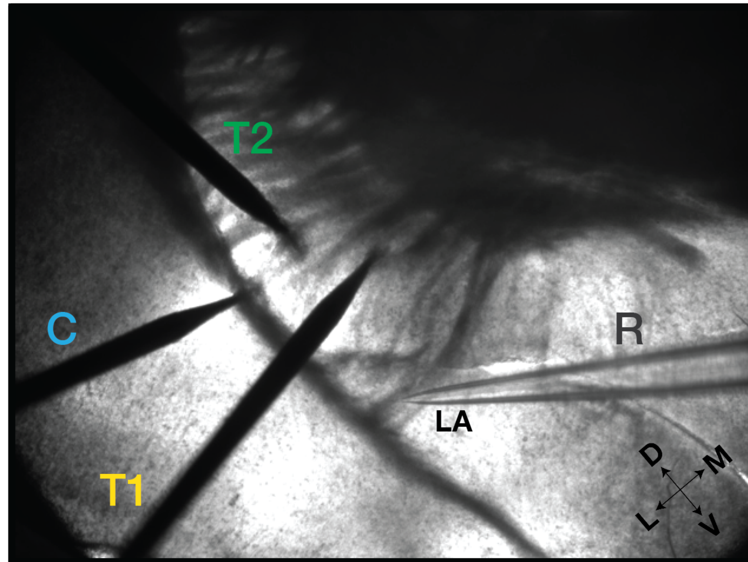
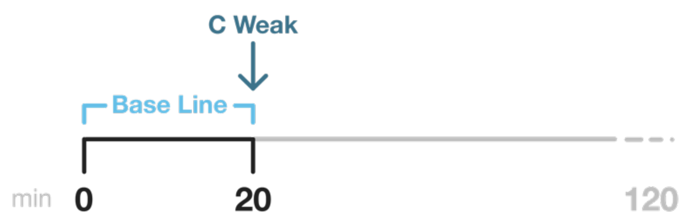


Figure 7: Positioning of the electrodes. Two stimulating electrodes are placed in afferent fibers of the internal capsule (T1 and T2) in order to stimulate the thalamic projections; the third stimulating electrode is placed in the external capsule (C) for cortical projections; the recording electrode is placed in the LA (R). Cross indicates orientation of the sections, in which L, M, D and V, respectively, stand for lateral, medial, dorsal and ventral.

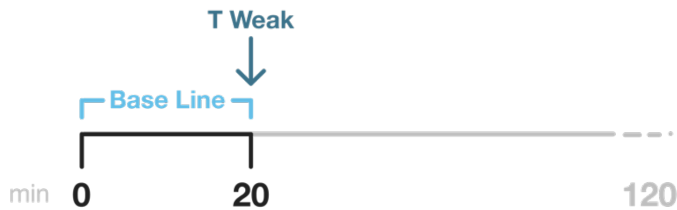
SYNAPTIC PLASTICITY INDUCTION

Initially, we induced transient forms of plasticity at cortical [**Scheme 1**] and thalamic inputs [**Scheme 2**] by weak tetanic stimulation (25 pulses at a frequency of 100 Hz, repeated 3 times, at an interval of 3 seconds). Thus, after recording a stable baseline of EPSPs for 20-min, weak stimulation was delivered either to the cortical (C Weak) or thalamic inputs (T Weak). In these experiments, we recorded only two pathways (one cortical and one thalamic), whereas only one pathway was stimulated. The non-stimulated inputs were used as control pathways.

Scheme 1 – Cortical weak stimulation (C Weak).

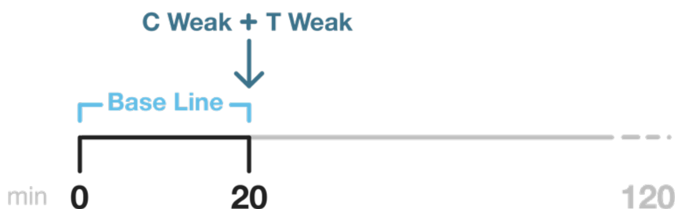


Scheme 2 – Thalamic Weak stimulation (T Weak).



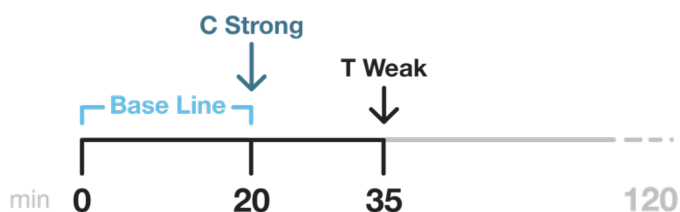
Then, we stimulated simultaneously the cortical (C Weak) and thalamic inputs (T Weak), with a weak tetanic stimulation [**Scheme 3**]. In these experiments, we also recorded only two pathways.

Scheme 3 – Coincident thalamic weak (T Weak) and cortical weak (C Weak) stimulation.



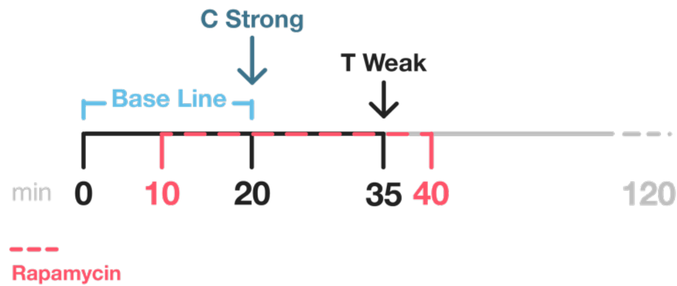
To test whether cortical and thalamic inputs engage in cooperation, we stimulated the cortical input with a strong stimulation protocol, and 15min after we stimulated with a weak stimulation protocol the thalamic input [**Scheme 4**]. Persistent forms of LTP in the cortical input were induced by strong tetanic stimulation of 25 pulses at a frequency of 100Hz, repeated 5 times, interval of 3 sec. In these experiments, a third stimulation electrode was placed in the internal capsule allowing us to record a third input (as a thalamic control pathway).

Scheme 4 – Cortical strong stimulation (C Strong) followed by thalamic weak stimulation (T Weak) 15-min after.



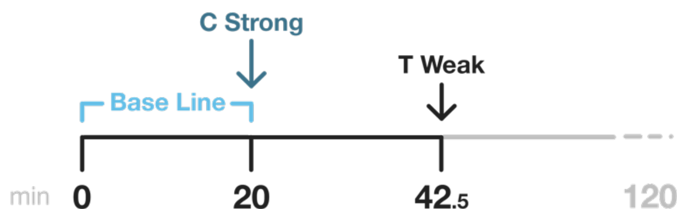
We repeated the experiment described above while applying rapamycin (a mTOR protein synthesis inhibitor) during a time window of 30-min [**Scheme 5**].

Scheme 5 – Cortical strong stimulation (C Strong) followed by thalamic weak stimulation (T Weak) 15-min after, while applying rapamycin.



We extended the time interval between the cortical strong stimulation (C Strong) and the thalamic weak stimulation (T Weak) up to 22,5-min [**Scheme 6**]. In these experiments, the second thalamic pathway served as a control.

Scheme 6 – Cortical strong stimulation (C Strong) followed by weak thalamic stimulation (T Weak) 22.5-min after.

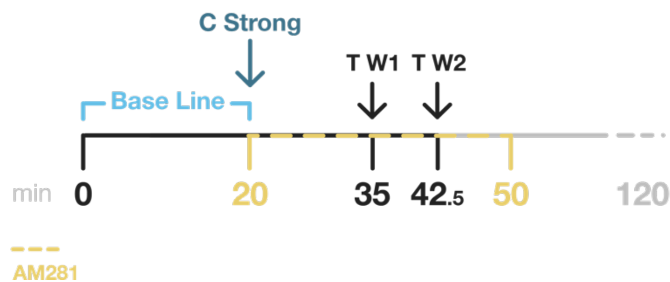


To examine if synaptic competition took place, we induced a transient form of LTP in a second thalamic projection (T W2), by a weak tetanic stimulation, 7.5-min after the stimulation of the first thalamic projection (T W1) [**Scheme 7**].

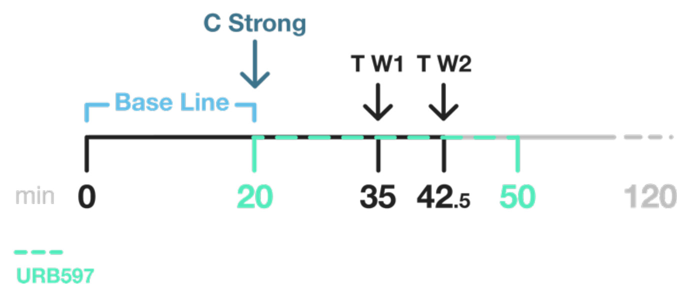
Scheme 7 – Cortical strong stimulation (C Strong) followed first by weak thalamic stimulation (T W1) 15-min after, then by a second thalamic weak stimulation (T W2) 7.5-min after the first thalamic.

In order to better understand the role of the eCB signaling in synaptic competition, we repeated the previous experiment while bath-applying AM281 (an inhibitor of the CB1R) and URB597 (an inhibitor of the enzyme FAAH) during 30min [**Scheme 8 and 9**]. A blinded trial was conducted in these experiments, in which for the control experiments [**Scheme 7**], only DMSO was added to the ACSF.

Scheme 8 – Cortical strong stimulation (C Strong) followed first by thalamic weak stimulation (T W1) 15-min after, then by a second thalamic weak stimulation (T W2) 7.5-min after the first thalamic, while applying AM281.

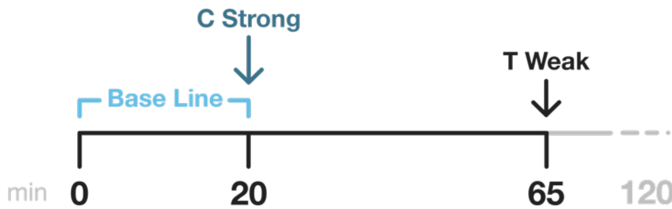


Scheme 9 – Cortical strong stimulation (C Strong) followed first by thalamic weak stimulation (T W1) 15-min after, then by a second thalamic weak stimulation (T W2) 7.5-min after the first thalamic, while applying URB597.



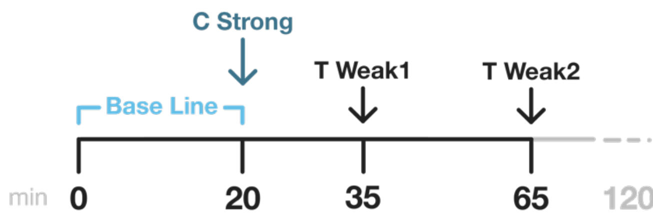
Next, we extended the time-window between the cortical strong stimulation (C Strong) and the thalamic weak stimulation (T Weak) to 45-min [**Scheme 10**].

Scheme 10 – Cortical strong stimulation (C Strong) followed by thalamic weak stimulation (T Weak) 45-min after.

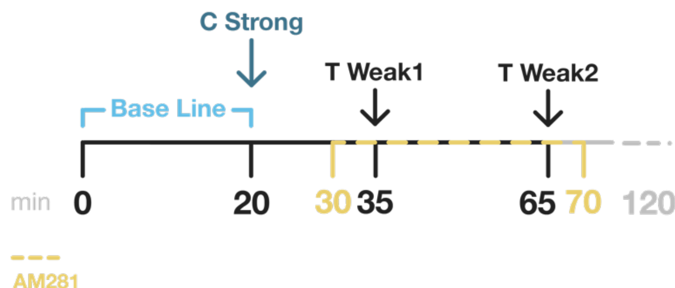


We stimulated a second thalamic input (T Weak2) through a weak stimulation protocol, 30-min after stimulating the first thalamic pathway (T Weak1) [**Scheme 11**]. We applied AM281 during 40-min [**Scheme 12**].

Scheme 11 – Cortical strong stimulation (C strong) followed first by thalamic weak stimulation (T Weak1) 15-min after, then by a second thalamic weak stimulation (T Weak2) 30-min after the first thalamic.

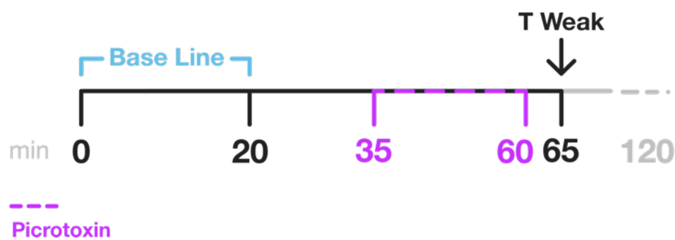


Scheme 12 – Cortical strong stimulation (C Strong) followed first by thalamic weak stimulation (T Weak1) 15-min after, then by a second thalamic weak stimulation (T Weak2) 30-min after the first thalamic, while applying AM281.

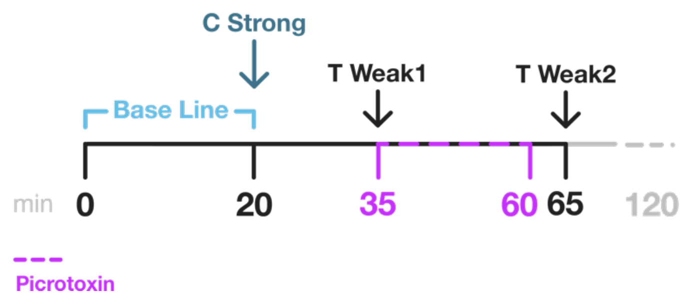


Next, we induced a transient form of LTP in the thalamic input (T Weak) 45-min after recording the baseline, and applied Picrotoxin (an antagonist of $GABA_A$ receptors) during 25-min [**Scheme 13**]. We repeated the experiment described in [**Scheme 11**] while bath-applying Picrotoxin during 25-min [**Scheme 14**].

Scheme 13 – Thalamic weak stimulation (T Weak) 45-min after the baseline, while applying Picrotoxin.

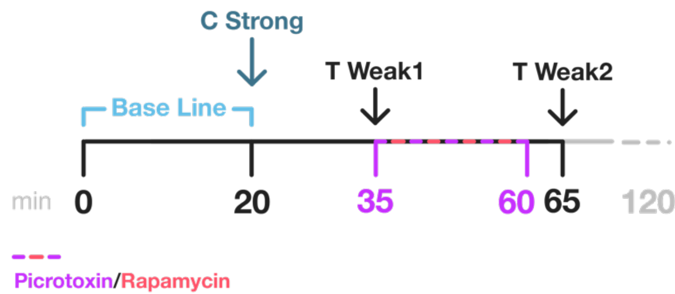


Scheme 14 – Cortical strong stimulation (C Strong) followed first by thalamic weak stimulation (T Weak1) 15-min after, then by a second thalamic weak stimulation (T Weak2) 30-min after the first thalamic, while applying Picrotoxin.



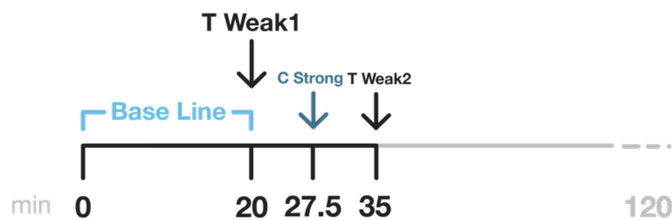
We also repeated the experiment in [**Scheme 11**] while bath-applying picrotoxin and rapamycin at the same time, for 25-min [**Scheme 15**].

<u>Scheme 15</u> – Cortical strong stimulation (C Strong) followed first by thalamic weak stimulation (T Weak1) 15-min after, then by a second thalamic weak stimulation (T Weak2) 30-min after the first thalamic, while applying picrotoxin and rapamycin.

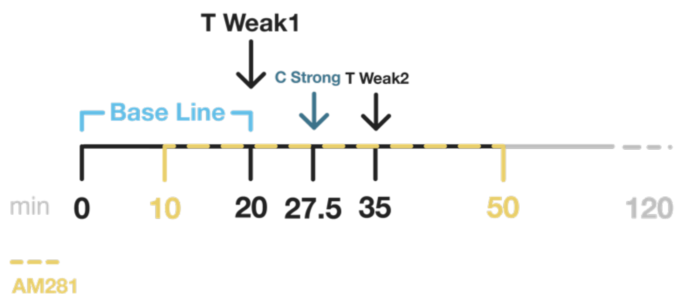


Then, we looked at the ability of a maintained form of cortical LTP induction to stabilize a transient form of LTP induced 7.5-min before, by stimulating one thalamic input with a weak stimulating protocol before the cortical input. Then, we stimulated the second thalamic input, with a weak stimulating protocol, 7.5-min after the cortical input [**Scheme 16**]. We bath-applied AM281 during 40min **[Scheme 17**].

<u>Scheme 16</u> – Thalamic weak stimulation (T Weak1) followed first by cortical strong (C Strong) 7.5-min after, then by thalamic weak stimulation (T Weak2) another 7.5-min after.



<u>Scheme 17</u> – Thalamic weak stimulation (T Weak1) followed first by cortical strong (C Strong) 7.5-min after, then by thalamic weak stimulation (T Weak2) another 7.5-min after, while applying AM281.



DRUG TREATMENT

The following drugs were dissolved in DMSO and diluted to achieve the final concentration: rapamycin (Tocris) 1 μM (in 0.01% DMSO), AM281 (Tocris) 0,5 μM (in 0.01% DMSO), URB597 (Tocris) 1 μM (in 0.01% DMSO) and picrotoxin (Sigma) 25 μM (in 0.01% DMSO). For the control experiments, only DMSO (in 0.01%) was added to the ACSF.

DATA ANALYSIS

Electrophysiological data were collected using a RK-400 amplifier (Bio-Logic, France) filtered at 1kHz and digitized at 10kHz using a Lab-PCI-6014 data acquisition board (National Instruments, Austin, TX) and stored on a PC. Offline data analysis was performed using a customized LabView-program (National Instruments).

As a measure for synaptic strength, the initial slope of the evoked EPSPs was calculated and expressed as percent changes from the baseline mean. Error bars denote SEM values. For the analysis LTP decay was calculated by $(T_{initial}-T_{final})/T_{initial} \times 100$, where $T_{initial}$ corresponds to the average of LTP values in the first 5-min after each stimulation (T1=20-25min; T2=27.5-32.5min; T3=35-40min; T4=42.5-47.5min; T5=65-70min), and T_{final}=T6=100-105min.

All experiments in which the control pathway decayed more than 20% were excluded. All experiments in which LTP induction failed (less than 20% increase above baseline) were also excluded.

To test for group differences between LTP decay values across tested conditions, first we confirmed normality (Shapiro-Wilk test) and homocedasticity (Levene test), then we performed a one-way ANOVA with a Tukey post-hoc test (Statistica StatSoft, DeLL software), at times $T_{initial}$ and T_{final} (100–105 min). For data sets that did not show a normal distribution, a non-parametric Kruskal-Wallis test was performed.

PPF values were obtained by dividing the slope of the second pulse by the slope of the first. PPF changes were calculated as percentage changes from baseline mean at 10, 25, 50 and 100ms interstimulus interval.

CHAPTER 4

RESULTS

SYNAPTIC COOPERATION BETWEEN CORTICAL AND THALAMIC INPUTS INTO LA NUCLEUS IS PROTEIN SYNTHESIS DEPENDENT

Recent work from our group determined the stimulation protocols capable of inducing transient and persistent forms of LTP at cortical and thalamic inputs (Fonseca, 2013). In these stimulation protocols, 100-Hz tetanic stimulations were used. We believe that this stimulation pattern represents the change in synaptic strength induced by fear-conditioning learning, resembling the activation of cortical and thalamic inputs to the LA (Kwon and Choi, 2009).

Our first approach was to confirm that we could induce transient forms of LTP with these same stimulation protocols. Interestingly, we had to increase the number of repetitions of the pulses from 2 to 3 times, to succeed in inducing transient forms of LTP. Thus, we induced transient forms of LTP, after recording a stable baseline of 20-min, by stimulating with a weak tetanus (25 pulses at a frequency of 100 Hz repeated 3 times, with a 3-sec interval) the thalamic or the cortical inputs. The non-stimulated inputs (cortical and thalamic, respectively) served as control pathways showing no decrement in synaptic transmission throughout the recording.

Weak stimulation of the thalamic or cortical inputs induced a transient form of LTP that decayed to baseline values within the recording (LTP T6 Thalamic Weak 114.0 ± 8.50% n=10, Cortical Control 95.6 ± 6.18% n=10; T2 Cortical Weak 117.4 ± 5.83% n=11, Thalamic Control 97.1 ± 3.43% n=11) [**Figure 9A and 9B**].

Next, in order to mimic the changes that occur in associative fear learning, and to take advantage of the associative properties of LTP in the LA, we decided to test whether concurrent activation of cortical and thalamic inputs results in persistent forms of LTP. We knew beforehand that weak tetanic stimulation results in transient forms of LTP, and strong stimulation in persistent forms of LTP, wherein the latter is related to the synthesis and allocation of PRPs. However, we decided to test whether concurrent weak stimulation of thalamic and cortical would lead to the reinforcement of both activated synapses groups. In other words, if transient forms of LTP can interact with each other, converting into persistent forms of LTP by protein synthesis and sharing.

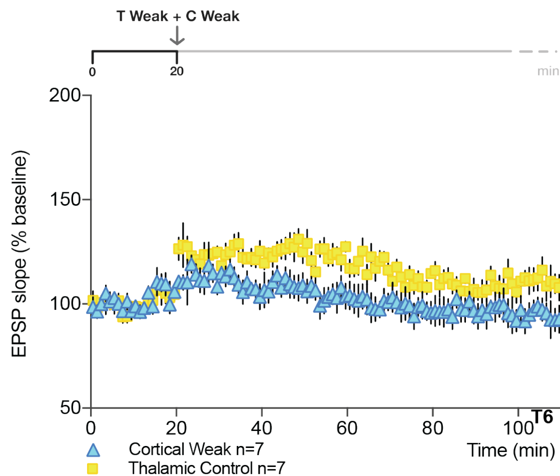


Figure 8: Co-activation of cortical and thalamic inputs does not result into persistent forms of LTP. Simultaneous induction of transient forms of LTP by weak stimulation of the cortical (△ C Weak) and thalamic (□ T Weak) input projections. Both stimulations led to the induction of transient forms of LTP (LTP T6 Cortical Weak 95.9 ± 5.60% n=7; Thalamic Weak 111.7 ± 5.14% n=7).

Our results show that this concurrent weak stimulation of thalamic and cortical inputs does not lead to stable and persistent forms of LTP (LTP T6 Cortical Weak 95.9 ± 5.60% n=7, Thalamic Weak 111.7 ± 5.14% n=7) [**Figure 8**].

Conversely, previous results from our laboratory demonstrated a form of associative plasticity in the LA, in which induction of transient forms of LTP, can be maintained by the capture of PRPs synthesized after an heterosynaptic strong LTP induction. In these experiments, a transient form of homosynaptic LTP at thalamic or cortical synapses is reinforced by a subsequent stimulation of a heterosynaptic input at cortical or thalamic inputs respectively (Fonseca, 2013). Weak stimulated synapses result into persistent forms of LTP since these tagged synapses are later able to capture PRPs synthetized upon strong stimulation of another pool of synapses. Therefore, the ability of these weak stimulated synapses to capture the PRPs is dependent on the duration of the tag. Specifically, the ability of the thalamic synapses to capture the PRPs synthesized upon the strong stimulation of cortical synapses is restricted to the duration of the tag, which in this case is restricted to a time window of 7.5-min between the two stimulations. While cortical synapses can capture the PRPs synthetized after the strong thalamic stimulation even within a 30-min interval. Since it would later be our goal to stimulate more than one pool of synapses, if we opted for this weak-before-strong configuration, we would be limited to the time-window that the synaptic tag lasts. To confirm that the thalamic (weak stimulated) synapses can cooperate with the cortical synapses leading to the induction of maintained forms of LTP, as a result of sharing the available PRPs synthetized after a strong tetanic stimulation, we decided to opt for a different approach.

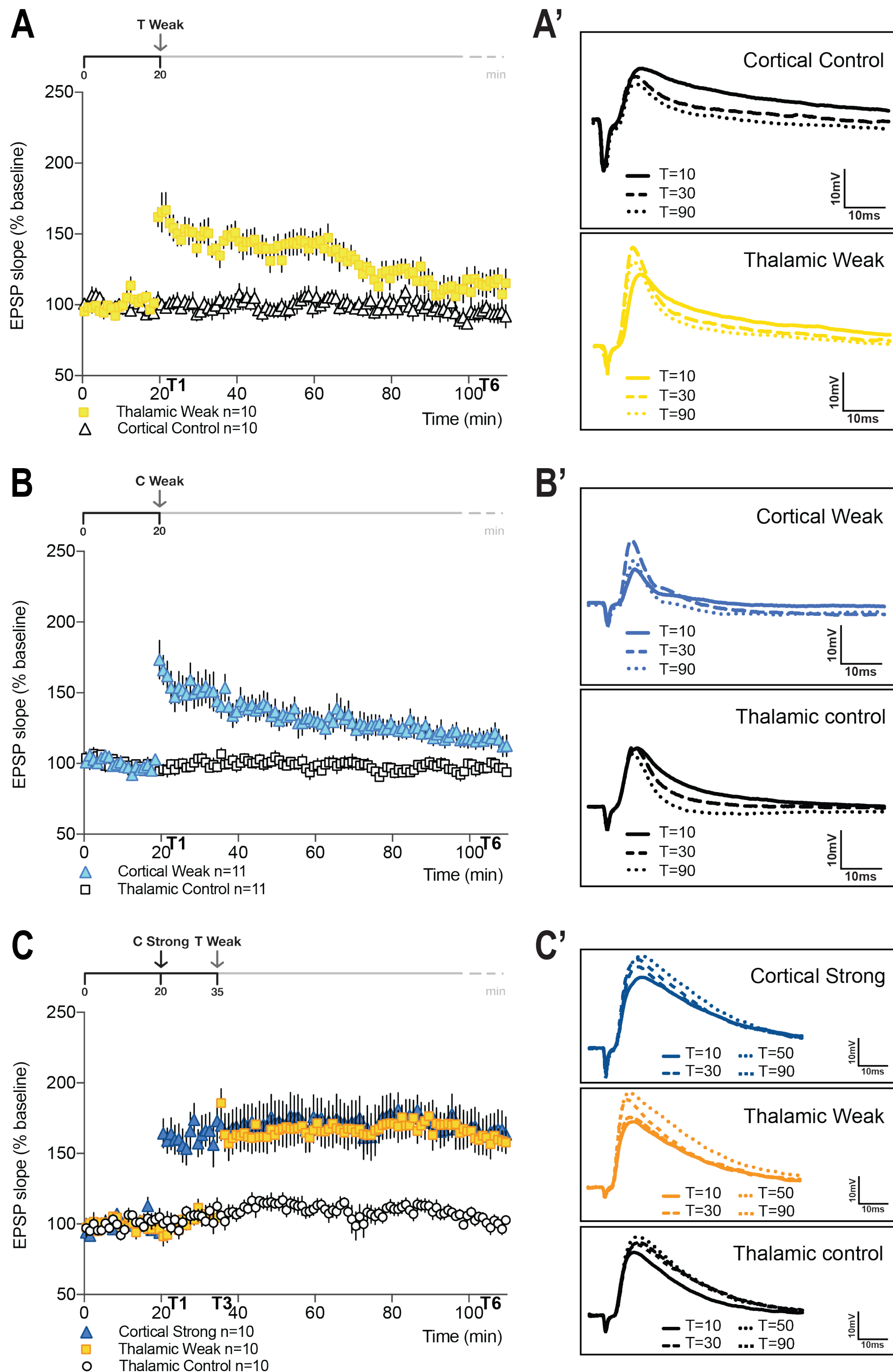


Figure 9: Cooperation between thalamic and cortical inputs to the lateral amygdala. (A) Weak tetanic stimulation (T Weak) leads to the induction of a transient LTP in the thalamic projection ( ) (LTP T6 Thalamic Weak 114.0 ± 8.50% n=10). No changes were observed in the non-stimulated

cortical projection (△) (T6 Cortical Control 95.6 ± 6.18% n=10). **(A')** Average of three EPSPs traces for cortical control and thalamic weak at 10-min, 30-min and 90-min. **(B)** Induction of a transient form of LTP in the cortical projection (△) with a weak tetanus (C Weak) (LTP T6 Cortical Weak 117.4 ± 5.83% n=11), while recording a non-stimulated thalamic projection ( ) (T6 Thalamic Control 97.1 ± 3.43% n=11). **(B')** Average of three EPSPs traces for thalamic control and cortical weak at 10-min, 30-min and 90-min. **(C)** Induction of a maintained form of LTP in the cortical projection (△), by strong cortical tetanic stimulation (C Strong) (LTP T6 Cortical Cooperation 165.6 ± 13.29% n=10), is able to convert a transient thalamic LTP into a maintained LTP (LTP T6 Thalamic Cooperation 162.0 ± 10.19% n=10). No changes were observed in a second thalamic projection (○) throughout the recorded time (T6 Thalamic Control 104.3 ± 3.71% n=10). **(C')** Average of four EPSPs traces for thalamic control, cortical strong and thalamic weak at 10-min, 30-min, 50-min and 90-min.

We stimulated the cortical input with a strong stimulation protocol followed by a thalamic weak stimulation. Strong stimulation of the cortical input was achieved with a strong tetanus (25 pulses at a frequency of 100 Hz repeated 5 times, with a 3-sec interval) that was maintained throughout the duration of the recording.

We found that weak thalamic stimulation results in the induction of a transient form of LTP, which can be converted into a persistent form of LTP by a previous strong stimulation of cortical input if this occur within 15-min. At the end of the recording, this associated plasticity led to the expression of persistent forms of LTP in both activated inputs. In these experiments, a third stimulation electrode was placed in the internal capsule allowing us to record a third input as a thalamic control pathway (LTP T6 Cortical Strong 165.6±13.29% n=10, Thalamic Weak 162.0±10.19% n=10, Thalamic Control 104.3±3.71% n=10) [**Figure 9C**].

Since this cortical-to-thalamic cooperation is based on PRPs sharing between tagged synapses, in which the strong cortical stimulation is the trigger for PRP synthesis, application of a protein synthesis inhibitor would block synaptic cooperation. Therefore, we repeated the cooperation design experiment while applying rapamycin (1 μM), a mTOR (mechanistic target of rapamycin) dependent protein synthesis inhibitor (Tang et al., 2002), during a time window of 40-min.

Rapamycin application was sufficient to block persistent forms of LTP, blocking synaptic cooperation between cortical and thalamic synapses (LTP T6 Cortical Strong Rapamycin 107.3 ± 5.88% n=11, Thalamic Weak Rapamycin 112.8 ± 8.78% n=11, Thalamic Control 96.2 ± 5.23% n=11) [**Figure 10A**]. Rapamycin had no effect on the viability of the

slices neither in the baseline transmission (no effect was seen in the thalamic control input throughout the recording).

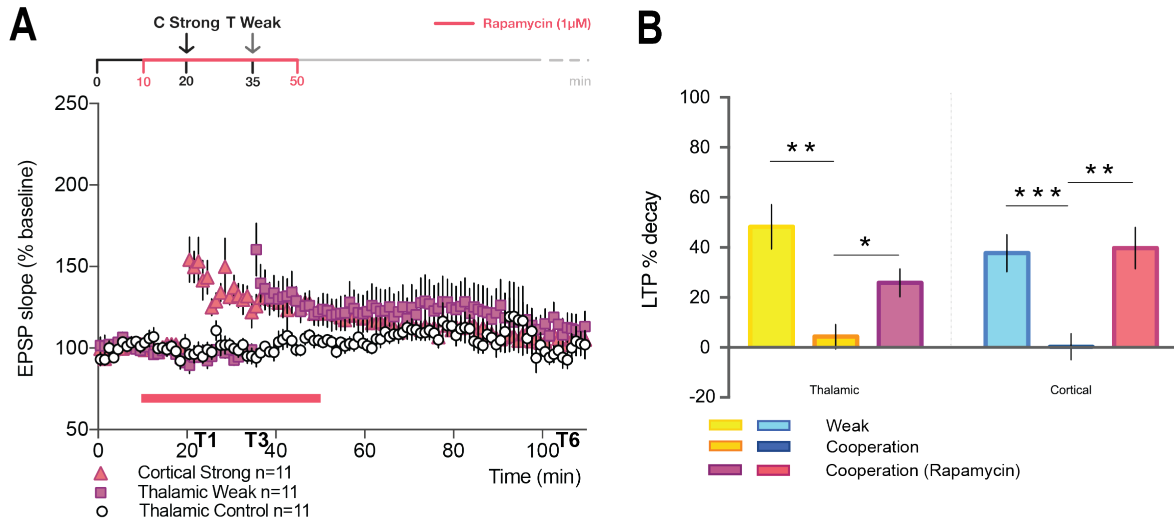


Figure 10: Cooperation is protein synthesis dependent. (A) Rapamycin application (1 µM) blocked synaptic cooperation between cortical and thalamic projections (LTP T6 Cortical Rapamycin 107.3 ± 5.88% n=11; Thalamic Rapamycin 112.8 ± 8.78% n=11; Thalamic Control 96.2 ± 5.23% n=11). **(B)** Summary plots showing the percentage decay for the time window $(T_{initial}-T_{final})/T_{initial}*100$. Both weak stimulation protocol and rapamycin application significantly increase LTP decay compared with the cooperation protocol in thalamic and cortical inputs. (Kruskal-Wallis test was performed; the only data set that did not show a normal distribution was: Cortical pathway in Figure 9 C; *p-value ≤ 0.05, **p-value ≤ 0.01 and ***p-value ≤ 0.001). n=number of slices.

Analysis of the LTP decay for the time window $(T_{initial}-T_{final})/T_{initial}*100$, showed that thalamic weak stimulation decayed significantly less if a cortical strong stimulation was induced previously. LTP decay in the thalamic input was also significantly higher when we applied rapamycin compared with the cooperation protocol (LTP % decay Thalamic Weak 48.2 ± 8.91% n=10, Thalamic Weak Cooperation 4.3 ± 4.95% n=10, Thalamic Weak Rapamycin 25.9 ± 5.56% n=11, H(2,32)=11.29 *p*=0.0035) [**Figure 10B**]. Regarding cortical inputs similar outcomes are observed, where both weak stimulation and rapamycin application significantly increase LTP decay compared with the cooperation protocol (LTP % decay Cortical Weak 37.7 ± 7.42% n=11, Cortical Strong Cooperation 0.3 ± 5.29% n=10, Cortical Strong Rapamycin 39.8 ± 8.33% n=11, H(2,31)=15.40 *p*=0.0005) [**Figure 10B**].

Together, these results indicate that cortical and thalamic inputs can cooperate within the time-window of 15-min in a protein-synthesis dependent fashion. This cortical-to-thalamic cooperation combines the presence of the activity of the “synaptic tag” induced by the thalamic transient LTP and the PRPs availability upon strong stimulation of the cortical input, within this time-interval between stimulations.

SYNAPTIC COMPETITION BETWEEN CORTICAL AND THALAMIC INPUTS INTO LA NUCLEUS

Just as we assume that there is cooperation, where PRPs are shared, competition between tagged synapses can also occur when the availability of these proteins is limited. Several studies have been describing this phenomenon, suggesting that this form of neuronal competition is critical for memory formation and neuronal allocation (Han et al., 2013; Yiu et al., 2014). Interestingly, electrophysiological findings report that synaptic competition can occur in the late stage of LTP when the availability of PRPs is reduced (Fonseca et al., 2004), as well as in the early-phase of synaptic memory consolidation culminating in a stable potentiated state or in its decay to baseline (Sajikumar et al., 2014).

Therefore, we aimed to examine if synaptic competition occurs in our cellular model by increasing the pool of activated synapses, and consequently the number of tags, to which PRPs are allocated. We induced a transient LTP in a second thalamic projection (T W2), by a weak tetanic stimulation, 7.5-min after the stimulation of the first thalamic projection (T W1) [**Figure 11C**]. This time-interval was defined since we knew, from previous results, that synapses interact with each other within 7.5-min (Fonseca, 2013), wherein this time-interval between weak thalamic and subsequent cortical strong stimulation (C Strong) significantly increases cooperation. Additionally, since cortical fibers reach the amygdala nuclei in a bundle (external capsule) we were not able to stimulate two independent cortical inputs and, therefore, a second thalamic input was defined as the additional pathway.

While cortical LTP was maintained during the recording, we observed that LTP in both thalamic inputs decayed to baseline (LTP T6 Thalamic 1 Competition 113.0 ± 7.61% n=10, Thalamic 2 Competition 109.6 ± 4.79% n=10, Cortical Competition 153.3 ± 12.09% n=10) [**Figure 11C**]. This suggests that weak activation of two distinct thalamic inputs within the temporal vicinity of strongly activated cortical synapses triggers an unbalance, wherein generated PRPs are insufficient to achieve long-term stabilization of all activated synapses. Interestingly, the strong stimulated input, the cortical pathway, behaves as a maintained form of LTP. We propose that these observations reveal a "winner-take-all" component of the competition phase of protein synthesis-dependent LTP in which the strongly potentiated synaptic population prevails to the detriment of the other weak activated synaptic populations.

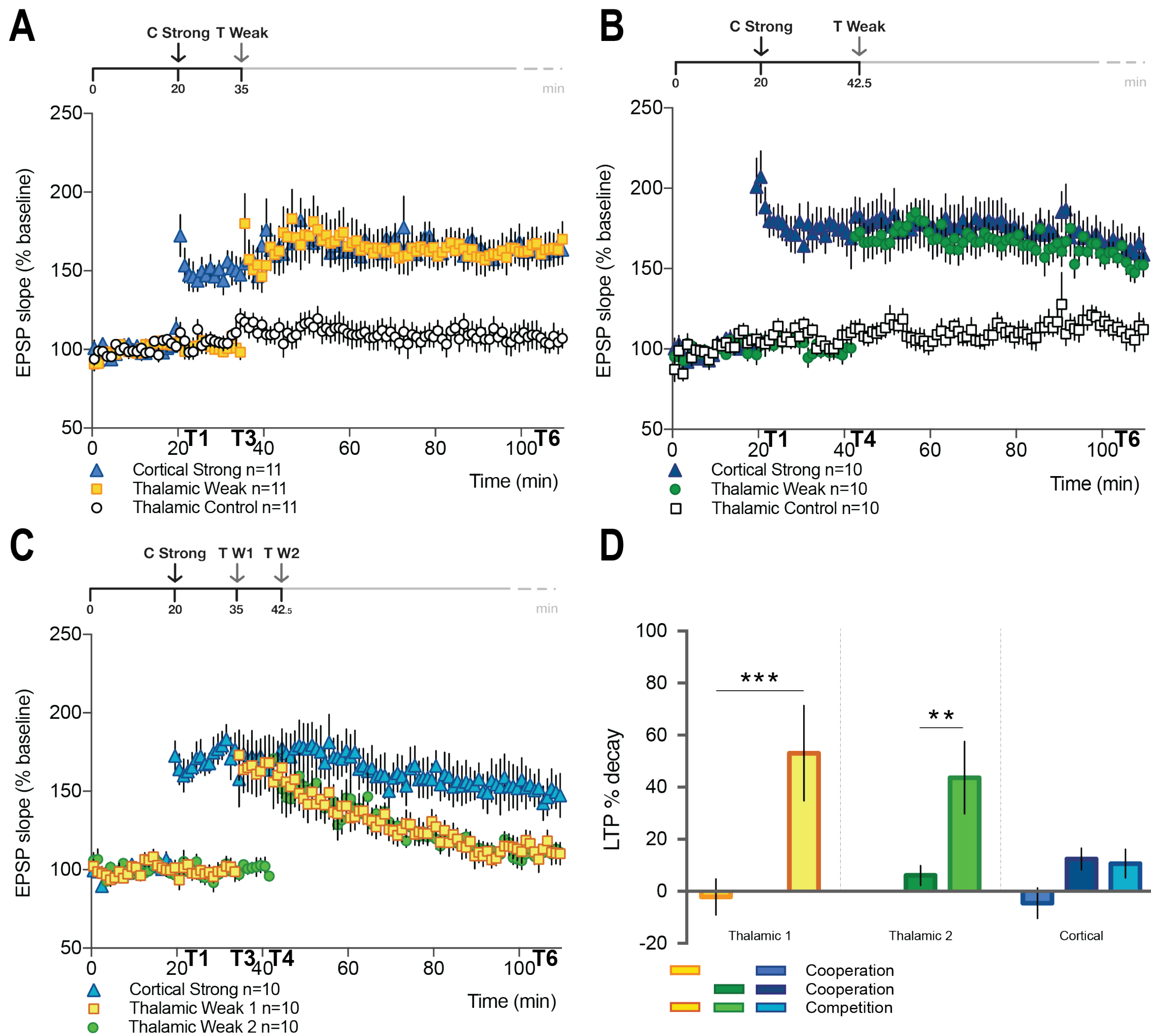


Figure 11: Competition between thalamic and cortical projections to LA pyramidal neurons. (A) Induction of a maintained form of LTP in the cortical projection (△), by cortical strong tetanic stimulation (C Strong) (LTP T6 Cortical Cooperation 163.5 ± 9.44% n=11), is able to convert a transient thalamic LTP into a maintained LTP (LTP T6 Thalamic Cooperation 163.8 ± 10.37% n=11). No changes were observed in a second thalamic projection (○) throughout the recorded time (T6 Thalamic Control 108.9 ± 8.29% n=11). (**B)** Synaptic cooperation occurs even extending the time interval between the cortical strong stimulation (△) (LTP T6 Cortical Cooperation 2 166.0 ± 10.80% n=10) and the weak thalamic stimulation ( ) by 22,5 min (LTP T6 Thalamic Cooperation 2 159.3 ± 6.72% n=10). No changes were observed in a second thalamic projection ( ) throughout the recorded time (T6 Thalamic Control 113.6 ± 6.55% n=10). (**C)** Induction of LTP in a second thalamic projection (○ T W2), by a weak tetanic stimulation 7.5-min after the stimulation of the first thalamic projection (T W1), leads to synaptic competition. LTP in thalamic projections (,○) returned to baseline during the recorded time (LTP T6 Thalamic 1 Competition 113.0 ± 7.61% n=10; Thalamic 2 Competition 109.6 ± 4.79% n=10), while cortical LTP was maintained (LTP T6 Cortical Competition 153.3 ± 12.09% n=10). **(D)** Summary plots showing the percentage decay for the time window ($T_{initial}$-T_{final})/$T_{initial}$*100 for the conditions tested. The experiments where a second thalamic input was

stimulated (Competition Protocol – in C), show a LTP decay significantly higher in both thalamic inputs than the cooperation experiments (A and C). There were no significant differences between LTP decay in cortical projections in the 3 conditions. (Kruskal-Wallis test was performed; the only data set that did not show a normal distribution was: Thalamic pathway 1 Competition in C; **p-value ≤ 0.01 and ***p-value ≤ 0.001). n=number of slices.

Since the second thalamic projection was stimulated 22.5-min after the cortical stimulation, we wanted to see if by extending the time interval up to 22.5-min between the cortical strong stimulation (C Strong) and the weak thalamic stimulation (T Weak), synapses still interact with each other and cooperation occurs [**Figure 11B**]. In these experiments, the second (non-stimulated) thalamic pathway served as a control. We found that synaptic cooperation occurs even extending the time interval to 22.5-min, proposing that PRPs are still available for allocation in thalamic synapses (LTP T6 Cortical Cooperation 2 166.0 ± 10.80% n=10, Thalamic Cooperation 2 159.3 ± 6.72% n=10, Thalamic Control 113.6 ± 6.55% n=10).

Statistical analysis of the percentage decay for all the conditions indicates that the experiment where a second thalamic input was stimulated, LTP decay was significantly higher in both thalamic inputs comparing with the cooperation experiments, in which thalamic inputs were stimulated at the same time interval after cortical stimulation [**Figure 11D**] (LTP % decay Thalamic Cooperation 2.2 ± 6.99% n=11, Thalamic 1 Competition 62.1 ± 18.57% n=10, $H(1,20)=10.92$ $p=0.0010$; Thalamic Cooperation 2 6.1 ± 3.88% n=10, Thalamic 2 Competition 62.2 ± 16.67% n=10, $H(1,19)=7.707$ $p=0.0055$). No significant differences were seen between LTP decay in cortical projections in cooperation and competition experiments (LTP % decay Cortical Cooperation 4.6 ± 5.87% n=11, Cortical Cooperation 2 12.4 ± 4.19% n=10, Cortical Competition 13.1 ± 5.88% n=10, $H(2,30)=5.14$ $p=0.0765$) [**Figure 11D**]. Together these results show that in a regime in which PRPs availability is limited and distinct pools of synapses are activated, a competitive tag is triggered, resulting in the persistence of the input with the strongest tag.

ENDOCANNABINOID SYSTEM PLAYS A ROLE IN SYNAPTIC COMPETITION

Earlier work from our group suggested that activation of the CB1R is involved in the time restriction of thalamic and cortical synaptic cooperation in an activity-dependent manner (Fonseca, 2013). Concretely, inhibition of CB1R extends the time window for cortico-to-thalamic cooperation. In other words, assuming that persistent forms of thalamic LTP require pre- and postsynaptic expression mechanisms (McKernan and Shinnick-Gallagher, 1997), the time interval between thalamic and cortical stimulations will define if the inhibitory effect of CB1R activation is effective in supressing the induction of presynaptic thalamic LTP (Shin et al., 2010), thus blocking the cortical-to-thalamic cooperation.

Longer time intervals (30-min) prevent thalamic tagged synapses to capture PRPs, since activity-dependent release of eCBs restricts the ability of thalamic synapses to benefit from the PRPs synthesized upon maintained forms of cortical LTP induction (Fonseca, 2013). Thus, we hypothesise that inhibition of CB1R increases the duration and strength of the tag.

Considering the above-mentioned, it is conceivable that pharmacological inhibition of CB1R can also modify the temporal rules of synaptic competition. To test this idea, we assessed competition by inducing a transient form of LTP, by a weak tetanic stimulation in the second thalamic input (T W2) after 7.5-min of the first thalamic stimulation, while bath applying AM281 (0.5 μM), an inhibitor of the CB1R, during a time window of 30-min.

Here we show that potentiation of a third pathway, when CB1R are blocked, triggers synaptic competition, preventing persistent forms of cortical and thalamic LTP (LTP T6 Cortical AM281 123.4 ± 11.16% n=10, Thalamic 1 AM281 119.4 ± 7.39% n=10, Thalamic 2 AM281 109.6 ± 4.79% n=10) [**Figure 12B**].

We postulate that in our experimental conditions, the inhibition of CB1R enables all three pathways, specifically the thalamic inputs, to be more excitable, strengthening the tag, thus to require higher amount of PRPs. Since PRPs are only produced by the strong cortical stimulation, the available amount will not be sufficient for all three activated pools of synapses, leading to the decrease of the LTP in cortical and thalamic inputs.

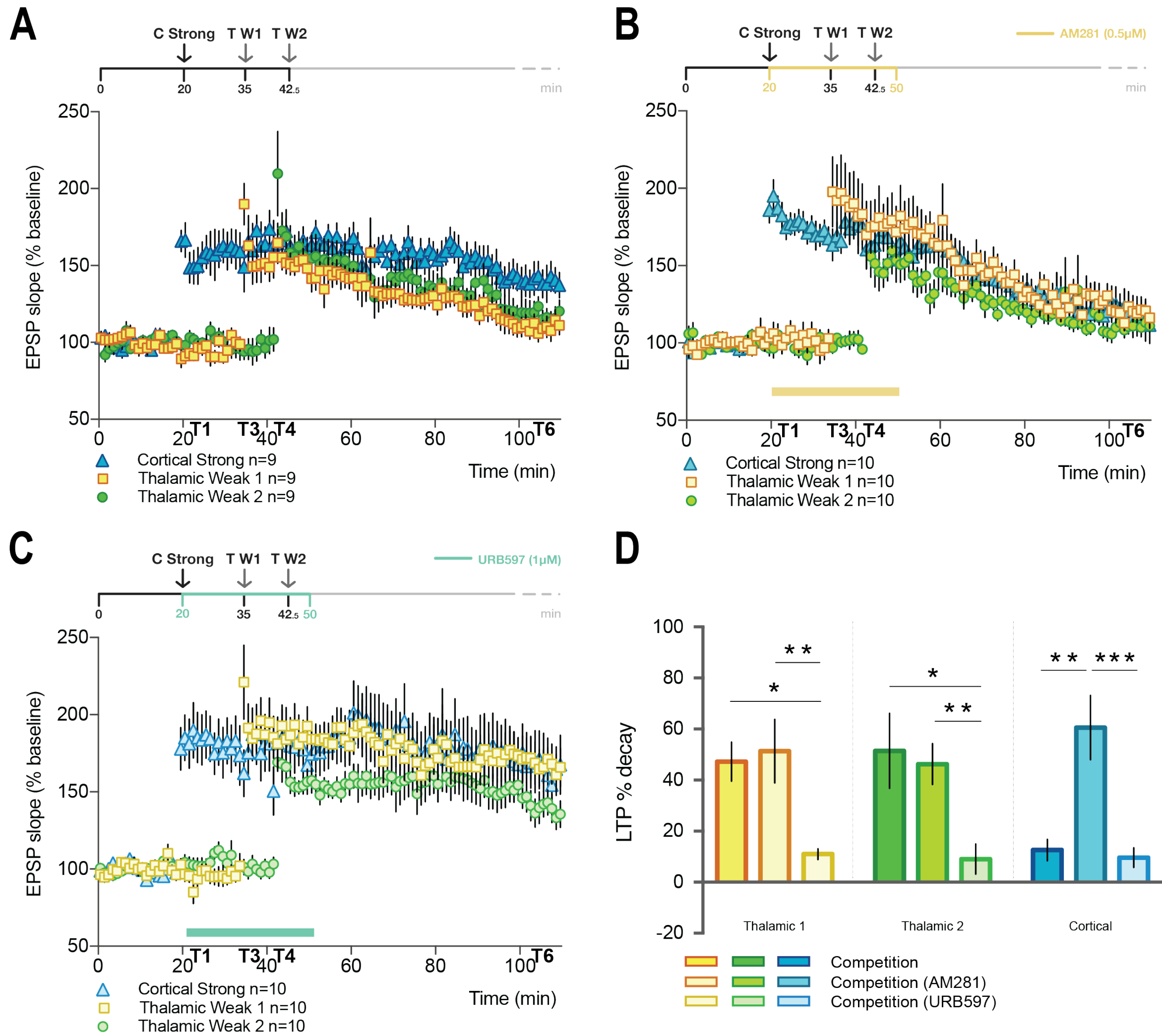


Figure 12: The endocannabinoid signalling also modulates synaptic competition. (A) Induction of LTP in a second thalamic projection (○ T W2), by a weak tetanic stimulation 7.5-min after the stimulation of the first thalamic projection (T W1), leads to synaptic competition. LTP in thalamic projections (,○) returned to baseline during the recorded time (LTP T6 Thalamic 1 Competition 109.4 ± 5.88% n=9; Thalamic Competition 2 120.1 ± 7.99% n=9). Cortical LTP was maintained during the recording (LTP T6 Cortical Competition 140.4 ± 10.06% n=9). **(B)** AM281 application (0.5 μM) increased synaptic competition between cortical and thalamic projections, blocking persistent forms of LTP in the 3 pathways (LTP T6 Thalamic 1 AM281 119.4 ± 7.39% n=10; Thalamic 2 AM281 109.6 ± 4.79% n=10; Cortical AM281 123.4 ± 11.16% n=10). **(C)** URB597 application (1 μM) completely abolished synaptic competition, leading to persistent forms of LTP in thalamic and cortical inputs (LTP T6 Thalamic 1 URB597 169.7 ± 14.55% n=10; Thalamic 2 URB597 142.0 ± 9.22% n=10; Cortical URB597 170.4 ± 18.42%). **(D)** Summary plots showing the percentage decay for the time window $(T_{initial}-T_{final})/T_{initial}*100$ for the conditions tested. Application of AM281, for 30min, leads to cortical LTP decay significantly higher than the other conditions. Application of URB597, at the same time interval of AM281, resulted in a decrease of the LTP decay in all pathways, comparing with the other conditions. (One-way ANOVA with a Tukey post-hoc test was performed; *p-value ≤ 0.05, **p-value ≤ 0.01 and ***p-value ≤ 0.001). n=number of slices.

Our *in vitro* data propose a link between CB1R activation and the restraining of cooperative interactions between the thalamic and cortical projections to the LA nucleus (Fonseca, 2013). Thus, we predicted that by increasing the availability of endocannabinoid ligands these restraining mechanisms would be enhanced. Curiously, URB597 application (1 µM) prevented synaptic competition, leading to persistent forms of LTP in thalamic and cortical inputs (LTP T6 Cortical URB597 170.4 ± 18.42% n=10, Thalamic 1 URB597 169.7 ± 14.55% n=10, Thalamic 2 URB597 142.0 ± 9.22% n=10) [**Figure 12C**]. URB597 is an inhibitor of the enzyme *fatty acid amide hydrolase* (FAAH) responsible for the degradation of fatty acid amides, one of which is the endocannabinoid anandamide. Therefore, application of this drug results in increased availability of endocannabinoids that bind to CB1 receptors. Since URB597 application completely abolished synaptic competition, prevailing cooperation, one possibility is that retrograde signaling of the eCBs, via activation of presynaptic CB1R, enables T W1 synapses to be more closed and stable. This state of stabilization leads to LTP maintenance and persistence during the recording, restricting competition. Consequently, T W2 synapses will have PRPs available for their maintenance and conversion into persistent forms of LTP.

Analysis of the LTP decay shows that AM281 application leads to cortical and thalamic LTP decay significantly higher than the other conditions. URB597 application results in a lower LTP decay in all pathways, comparing with the other conditions [**Figure 12D**] (LTP % decay Thalamic 1 Competition 47.2 ± 7.68% n=9, Thalamic 1 AM281 51.4 ± 12.51% n=10, Thalamic 1 URB597 11.0 ± 2.15% n=10, $F(2,26)=6.74$ $p=0.0044$; Thalamic 2 Competition 51.5 ± 14.75% n=9, Thalamic 2 AM281 46.3 ± 8.05% n=10, Thalamic 2 URB597 9.1 ± 5.94% n=10, $F(2,26)=5.50$ $p=0.0102$; Cortical Competition 12.6 ± 4.24% n=9, Cortical AM281 60.6 ± 12.67% n=10, Cortical URB597 9.6 ± 3.87% n=10, $F(2,26)=12.27$ $p=0.0002$).

INHIBITION OF THE CB1 RECEPTORS STRENGTHS THE TAG AND EXTENDS THE TIME WINDOW FOR SYNAPTIC COMPETITION

As mentioned formerly, the STC hypothesis proposes a cellular mechanism that enables the association of events separated in time (Redondo et al., 2010). Moreover, since LTP can be divided in several stages or phases, this opens the possibility for synapses to interact cooperatively and competitively in all these time periods (Reymann and Frey, 2007). With this line of thought we wanted to better characterize the time-related constraints of cooperation and competition between cortical and thalamic inputs.

We tested whether competition took place, after stimulating a second thalamic input (T Weak2), through a weak stimulation, 30-min after stimulating the first thalamic pathway (T Weak1) [**Figure 13A**].

We found that a transient form of LTP, induced by weak stimulation of the first thalamic input (T Weak1), was converted into a persistent form of LTP by the previous induction of a strong cortical stimulation. The second thalamic input (T Weak2), stimulated with a weak tetanus 45-min after the cortical strong stimulation (C Strong), was not converted into a maintained LTP (LTP T6 Thalamic 1 Competition 2 177.8 ± 17.87% n=9; Thalamic 2 Competition 2 148.3 ± 8.10% n=9; Cortical Competition 2 168.0 ± 11.56% n=9). Although the LTP values have not decreased to values as close to the baseline as in previous experiments, we believe that by extending the duration of the recording, this would occur. The presence of a maintained form of LTP in cortical and thalamic 1 inputs and transient LTP in the thalamic 2 input opened one possibility: C Strong and T Weak1 inputs cooperate with each other by sharing the PRPs, resulting in the stabilization and consolidation of the thalamic pathway, and T Weak2 input does not have enough PRPs available for its stabilization. Since T Weak2 is stimulated 30-min after, T Weak1 at that time is consolidated and no longer require PRPs. This can explain why T Weak1 is no longer interfered by the T Weak2 stimulation.

To test whether T Weak2 input is no longer capable to stabilize into a long-lasting form of LTP since the time-interval between the C Strong is longer, we extended the time-window between the cortical strong stimulation (C Strong) and the thalamic weak stimulation (T Weak) to 45-min. A second (non-stimulated) thalamic pathway served as a control

[**Figure 13B**].

We found that a transient LTP, induced by a weak stimulation of the thalamic input, is not converted into a persistent LTP by the C Strong input if the interval between stimulations is 45-min (LTP T6 Cortical Cooperation 3 155.9 ± 6.93% n=10; Thalamic Cooperation 3 127.3 ± 6.14% n=10; Thalamic Control 106.8 ± 4.59% n=10).

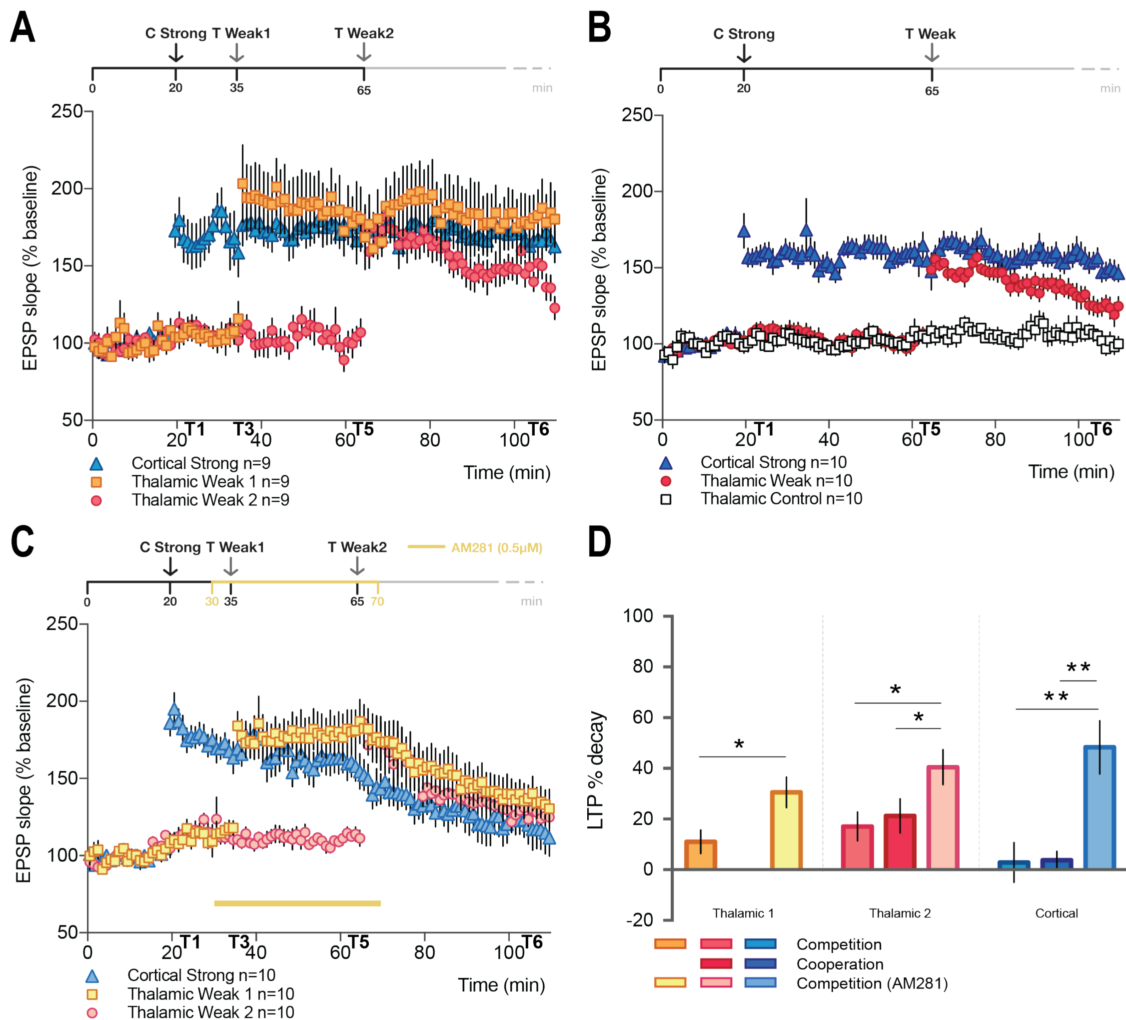


Figure 13: Inhibition of the CB1 receptors can extend the time-window of competition between thalamic and cortical inputs. (A) Induction of LTP in a second thalamic projection (○ T Weak2), by a weak tetanic stimulation, 30-min after the stimulation of the first thalamic projection (T Weak1), resulted in a transient form of LTP in T Weak2 (LTP T6 Thalamic 2 Competition 2 148.3 ± 8.10% n=9). LTP in the first thalamic projection weak stimulated (   ) and in the cortical projection strong stimulated (△) were maintained throughout the recorded time (LTP T6 Thalamic 1 Competition 2

177.8 ± 17.87% n=9; Cortical Competition 2 168.0 ± 11.56% n=9). **(B)** Induction of a maintained form of LTP in the cortical projection (△), by strong cortical tetanic stimulation (C Strong) (LTP T6 Cortical Cooperation 3 155.9 ± 6.93% n=10), is not able to convert a transient thalamic (○) LTP into a maintained LTP after 35-min (LTP T6 Thalamic Cooperation 3 127.3 ± 6.14% n=10). No changes were observed in a second thalamic projection ( ) throughout the recorded time (T6 Thalamic Control 106.8 ± 4.59% n=10). **(C)** AM281 (0.5 μM) was applied starting at 30-min and washout 5-min after T Weak2 (total 40-min). Application of AM281 extended the time-window for competition between cortical and thalamic projections, blocking persistent forms of LTP in the 3 pathways (LTP T6 Thalamic 1 AM281 137.6 ± 10.14% n=10; Thalamic 2 AM281 126.4 ± 5.17% n=10; Cortical AM281 123.4 ± 11.16% n=10). **(D)** Summary plots showing the percentage decay for the time window ($T_{initial}$-T_{final})/$T_{initial}$*100 for the conditions tested. AM281 application resulted in a significant higher LTP decay in the cortical and thalamic inputs. (One-way ANOVA with a Tukey post-hoc test was performed; *p-value ≤ 0.05 and **p-value ≤ 0.01). n=number of slices.

Since by extending the time between the T Weak1 and the T Weak2 stimulation to 30-min the LTP induced in T Weak1 is maintained, we wanted to better characterize the importance of CB1R activation in synaptic competition. Thus, we applied AM281 (0.5 μM) during 40-min, starting 5-min before T Weak1 stimulation (this is the time that the drug takes to get to the chamber), and ending 5-min after T Weak2 activation [**Figure 13C**]. We discovered that when CB1R are blocked, synaptic competition between cortical and thalamic synapses is triggered, preventing maintained forms of LTP (LTP T6 Thalamic 1 AM281 137.6 ± 10.14% n=10; Thalamic 2 AM281 126.4 ± 5.17% n=10; Cortical AM281 123.4 ± 11.16% n=10). Since whenever we apply AM281, LTP in all pathways decreases, we can deduce that its application is sufficient to strength the tag in the thalamic synapses, resulting in synaptic competition for the PRPs available.

Statistical analysis of LTP decay shows that inhibition of the CB1R, by AM281 application, when we extend the time-window between thalamic stimulations, results in a significant higher LTP decay in the cortical and thalamic inputs [**Figure 12D**] (LTP % decay Thalamic 1 Competition 2 11.1 ± 4.68% n=9, Thalamic 1 AM281 30.5 ± 6.09% n=10, $F(1,17)=6.21$ $p=0.0233$; Thalamic Cooperation 3 21.2 ± 6.81% n=10, Thalamic 2 Competition 2 17.1 ± 5.73% n=9, Thalamic 2 AM281 40.5 ± 6.99% n=10, $F(2,26)=3.61$ $p=0.0413$; T2 Cortical Cooperation 3 3.8 ± 3.62% n=10, Cortical Competition 2 2.8 ± 7.92% n=9, Cortical AM281 48.3 ± 10.56% n=10, $F(2,26)=10.96$ $p=0.0004$).

Next, to rule out the effect of AM281 application under baseline conditions, we analysed input-output (I/O) curves and pair-pulse facilitation (PPF) in AM281-treated slices [**Figure 14**]. No significant change was detected before and after drug application at cortical

and thalamic inputs. We did not observe CB1R blockage contributing to basal synaptic transmission in thalamic and cortical inputs as application of AM281 had no impact on baseline EPSP slope (Mahanty and Sah, 1999) [**Figure 14A, B**]. Despite the fact that PPF ratio is exceptionally induced by postsynaptic mechanism (Wang and Kelly, 1997), it is generally accepted that changes in PPF reflect a modulation of the presynaptic release probability. We found that AM281 application does not change PPF in cortical and thalamic inputs [**Figure 14C, D**].

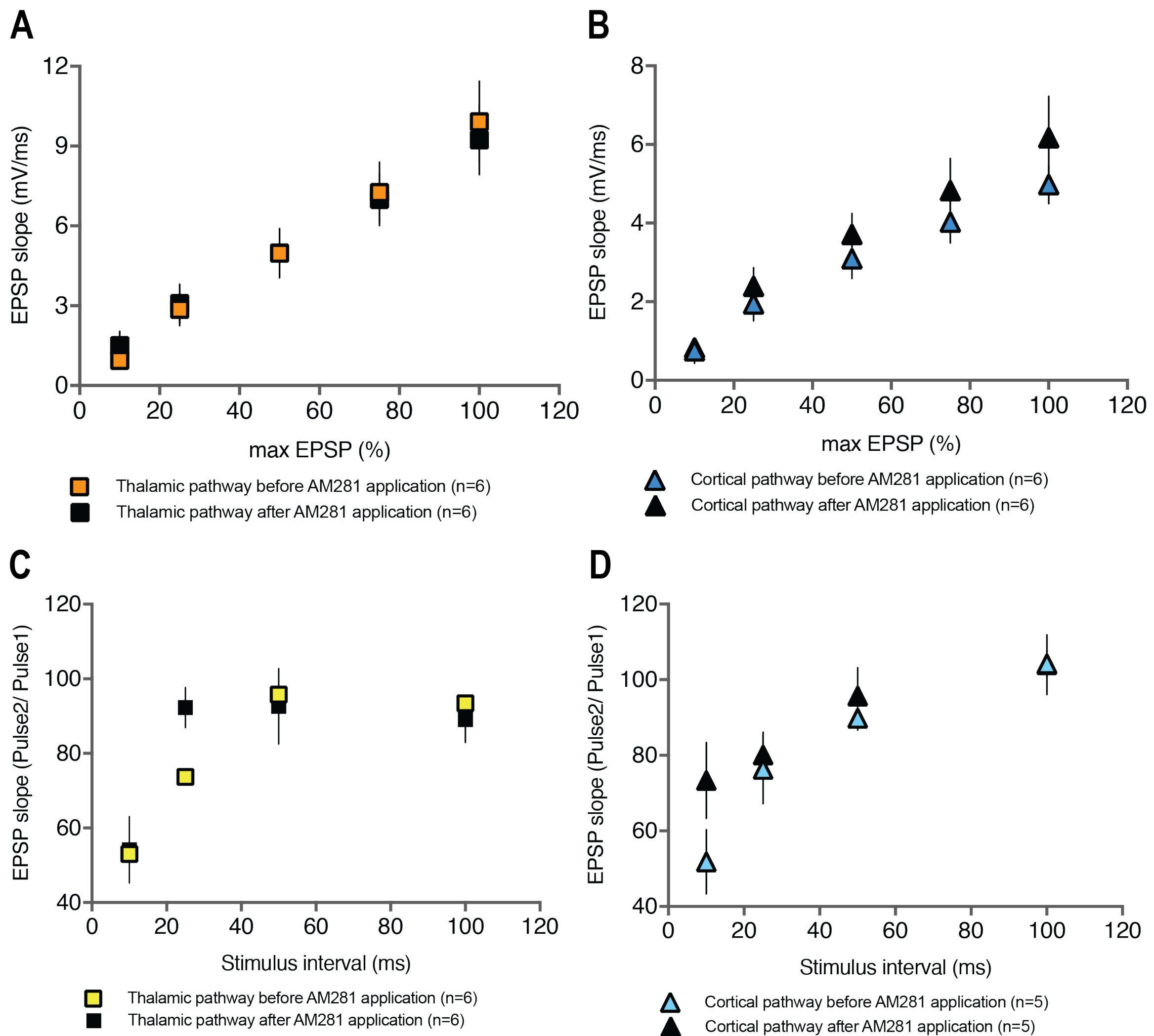


Figure 14: (A and B) I/O curve for thalamic and cortical inputs before and after AM281 application. The drug was bath-applied for 30-min similarly to the electrophysiology experiments. EPSP slope was measured by increasing steps of current (10, 25, 50, 75 and 100 % of the maximum EPSP) before and after drug application. No significant difference was obtained for the conditions tested. Data point correspond to the average of three consecutive pulses for each value of injected

current averaged crossed experiments. **(C and D)** AM281 did not alter PPF in thalamic and cortical inputs, as assessed by EPSP responses evaluated at 10, 25, 50 and 100-ms interstimulus interval. Data points correspond to the average of three consecutive pulses for each value of injected current averaged crossed experiments.

INHIBITION OF THE $GABA_A$ RECEPTOR ENHANCES SYNAPTIC COOPERATION IN A PROTEIN SYNTHESIS DEPENDENT FASHION

There is plentiful *in vivo* and *in vitro* data showing the existence of a strong inhibitory component in the LA (Danober and Pape, 1998; Lang and Paré, 1998; Pape and Pare, 2010). Indeed, GABAergic interneurons are thought to play an essential role in information processing in the LA (Mahanty and Sah, 1999). Interestingly, the induction of LTP in principal LA neurons is able to suppress inhibition from local interneurons (Li and LeDoux, 1995).

Recently it was found that a competitive process governs the interaction between engrams to distinguish memories for events happening farther apart in time and integrate memories for events occurring shortly in time (Rashid et al., 2016). This form of competition is a reflection of excitatory-inhibitory balance, and determines whether memories are segregated or bound in the LA (Rashid et al., 2016). This is consistent with what is known about neural networks, where winner-take-all competition is regulated by excitation and inhibition (Shoemaker, 2015).

Given all this we decided to test if inhibition plays a role in synaptic competition between thalamic and cortical inputs to the LA, by assessing whether $GABA_A$ inhibition alters competitive interactions. Since we did not want to alter the threshold for LTP induction we tested in the competition protocol with the biggest time window between stimulations (30-min interval between thalamic inputs).

First we assured that Picrotoxin (a $GABA_A$ receptor pore-blocker) application (Inomata et al., 1988), did not change the performance of a transient LTP after a thalamic weak stimulation (T Weak). Therefore we applied Picrotoxin during 25-min, starting the washout 5-min before the stimulation of the thalamic input [**Figure 15B**]. We have optimized the concentration of Picrotoxin to 25 μM so that transient thalamic LTP could result indeed in a transient LTP. We found that application of Picrotoxin (25 μM) does not change transient LTP induction in the thalamic input (LTP T6 Thalamic Weak Picrotoxin 134.8 ± 7.37% n=9; Thalamic Control Picrotoxin 111.5 ± 10.91% n=9; Cortical Control Picrotoxin 119.2 ± 8.98% n=9).

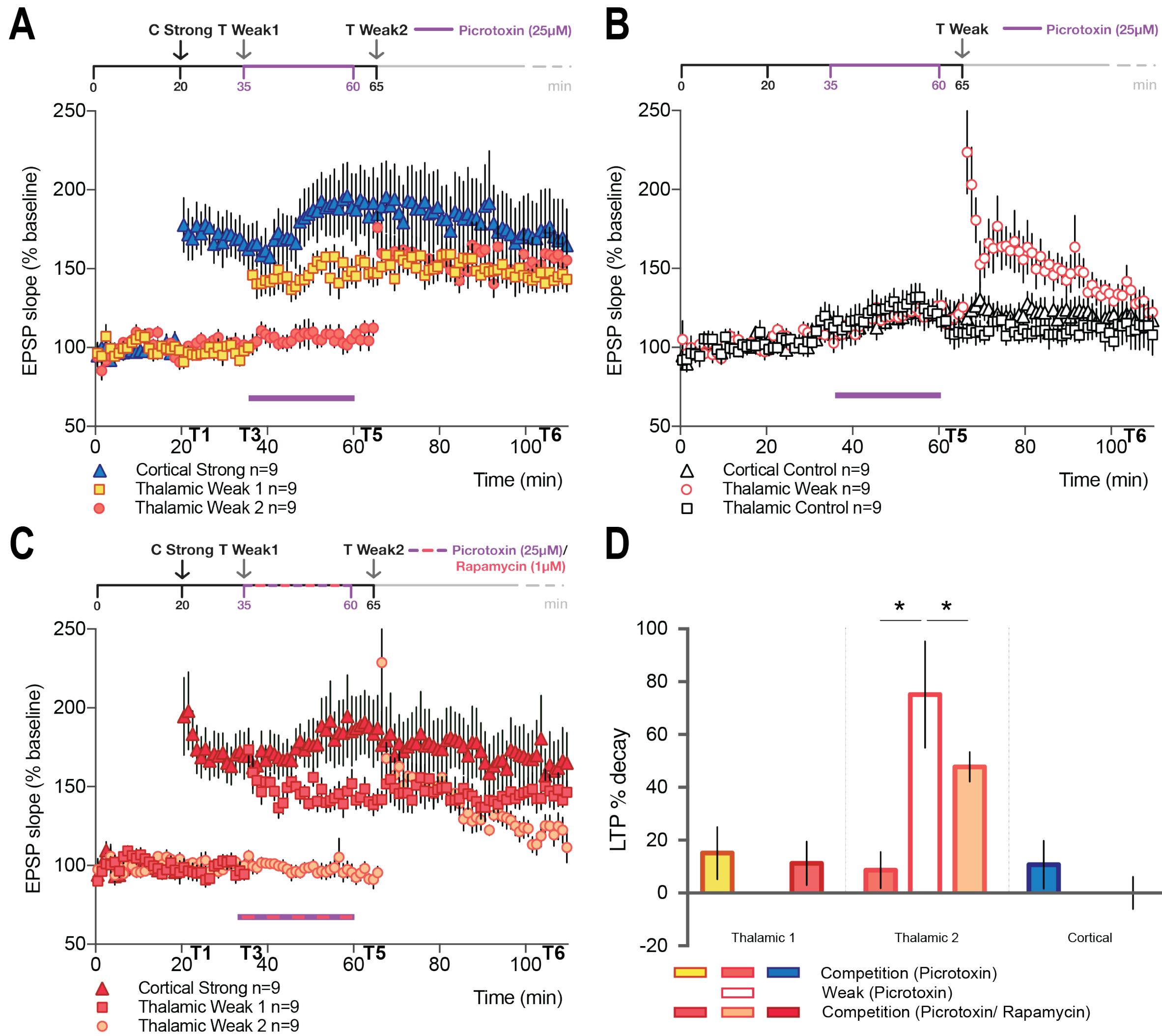


Figure 15: Synaptic cooperation is enhanced by $GABA_A$ receptor inhibition in a protein synthesis dependent fashion. (A) Induction of LTP in a second thalamic projection (○ T Weak2), by a weak tetanic stimulation 30-min after the stimulation of the first thalamic projection (T Weak1), while bath-applying Picrotoxin (25 μM) during 25-min, resulted in maintained forms of LTP in cortical, T Weak1 and T Weak2 (△, ,○) throughout the recorded time (LTP T6 Thalamic 1 Picrotoxin 144.0 ± 6.99% n=9; Thalamic 2 Picrotoxin 153.6 ± 15.54% n=9; Cortical Picrotoxin 171.9 ± 24.52% n=9). **(B)** Induction of a transient LTP in the thalamic input at 65-min, after bath-applying Picrotoxin (25 μM), starting at 35-min and washout 5-min before T Weak (25-min total) (LTP T6 Thalamic Weak Picrotoxin 134.8 ± 7.37% n=9). No changes were observed in the control pathways (△,) throughout the recorded time (T6 Thalamic Control Picrotoxin 111.5 ± 10.91% n=9; Cortical Control Picrotoxin 119.2 ± 8.98% n=9). **(C)** Applying picrotoxin (25 μM) and rapamycin (1 μM) resulted in a transient form of LTP in the second thalamic input weak stimulated (LTP T6 Thalamic 1 Picrotoxin/ Rapamycin 146.1 ± 9.31% n=9; Thalamic 2 Picrotoxin/ Rapamycin 118.8 ± 6.23% n=9; Cortical Picrotoxin/ Rapamycin 168.5 ± 20.09% n=9). **(D)** Summary plots showing the percentage decay for the time window ($T_{initial}$-T_{final})/$T_{initial}$*100 for the conditions tested. Bath-applying Picrotoxin in the condition B resulted in a significant decrease in the LTP decay in the thalamic pathway 2, comparing with the

other conditions. Simultaneous application of picrotoxin and rapamycin show a significant lower LTP decay in the thalamic pathway 2, comparing with the condition A, and a significant higher LTP decay when compared with the condition B. (Kruskal-Wallis test was performed; the only data set that did not show a normal distribution was: Thalamic pathway 2 in C; *p-value ≤ 0.05). n=number of slices.

We then looked whether synaptic competition took place after weak stimulating the T Weak2 30-min after stimulating T Weak1, while applying Picrotoxin during 25-min starting right after T Weak1 stimulation and ending 5-min before T Weak2 activation [**Figure 15A**]. Interestingly, we found that blocking $GABA_A$ receptors prevents competition or, in other words, enhances synaptic cooperation, since both thalamic inputs are converted into maintained forms of LTP by the C Strong input (LTP T6 Thalamic 1 Picrotoxin 144.0 ± 6.99% n=9; Thalamic 2 Picrotoxin 153.6 ± 15.54% n=9; Cortical Picrotoxin 171.9 ± 24.52% n=9).

We decided to test whether or not this enhancement was due to to the treshold modifications resulting from the $GABA_A$ receptors blockage, or the increase of PRPs availability. Thus, we co-applied rapamycin (1 µM) and Picrotoxin (25 µM) at the same time-window [**Figure 15C**]. We found that this enhancement of synaptic cooperation is protein-synthesis dependent, since rapamycin application resulted in a transient form of LTP at the second thalamic input weak stimulated (LTP T6 Thalamic 1 Picrotoxin/ Rapamycin 146.1 ± 9.31% n=9; Thalamic 2 Picrotoxin/ Rapamycin 118.8 ± 6.23% n=9; Cortical Picrotoxin/ Rapamycin 168.5 ± 20.09% n=9).

Statistical analysis of LTP decay, shows that simultaneous application of picrotoxin and rapamycin results in a significant lower LTP decay in the T Weak2, comparing with the condition where only one thalamic input was stimulated while applying Picrotoxin. Moreover, a significant higher LTP decay was observed after co-application of picrotoxin and rapamycin when compared with the condition Competition 2 in which Picrotoxin was bath-applied. (LTP % decay Weak Picrotoxin 75.2 ± 20.20% n=9, Thalamic 2 Picrotoxin 8.7 ± 6.97% n=9, Thalamic 2 Picrotoxin/ Rapamycin 47.8 ± 5.63% n=9, $H(2,27)=9.36$ $p=0.0093$; Thalamic 1 Picrotoxin 15.1 ± 9.92% n=9, Thalamic 1 Picrotoxin/ Rapamycin 11.3 ± 8.29% n=9, $H(1,18)=0.33$ $p=0.57$). No significant differences were seen between LTP decay in cortical inputs (LTP % decay Cortical Picrotoxin 10.7 ± 9.16% n=9, Cortical Picrotoxin/ Rapamycin 0.02 ± 6.19% n=9, $H(1,18)=0.10$ $p=0.7573$).

SYNAPTIC COMPETITION BETWEEN THALAMIC AND CORTICAL INPUTS INTO LA DEPENDS ON THE REDUCED AVAILABILITY OF PRPS

Our results show that synaptic competition occurs when we stimulate the first thalamic pathway (T Weak1), with a weak tetanus, 15-min after the cortical input strong stimulated (C Strong), followed by another thalamic input weak stimulated (T Weak2) with a 7.5-min interval. In a subsequent approach, we decided to test whether competition would occur if one of the thalamic pathways was stimulated before the cortical pathway.

Since previous studies from our group show that thalamic synapses can capture the PRPs, i.e. that their tag is maintained within the time interval of 7.5-min (Fonseca, 2013), we induced a transient form of LTP in a thalamic pathway (T Weak1) 7.5min before the cortical pathway (C Strong). We then induced a transient form of LTP in another thalamic pathway (T Weak2) 7.5-min after C Strong [**Figure 16A**]. We have verified that instead of synaptic competition, synaptic cooperation occurs, in which the two thalamic inputs capture the PRPs produced by the cortical pathway, inducing persistent forms of plasticity (LTP T6 Cortical Competition 3 147.2 ± 8.02% n=9; Thalamic 1 Competition 3 166.9 ± 19.34% n=9; Thalamic 2 Competition 3 139.5 ± 6.27% n=9). Thus, we can assume that the two distinct pools of thalamic inputs have enough PRPs available, allowing them to remain stable and persistent throughout the entire recording.

We have shown that whenever we apply AM281, the LTP of all pathways decay to the baseline. Hence, we decided to see what would happen in this configuration. We applied AM281, also in a time window of 40-min, starting 10-min before the stimulation of first thalamic input [**Figure 16B**]. Once again, we saw that blocking CB1R resulted in a transient form of plasticity in all thalamic and cortical inputs, that decayed to baseline values within the 110-min of the recording (LTP T6 Thalamic 1 AM281 123.3 ± 5.59% n=10; Thalamic 2 AM281 124.4 ± 5.18% n=10; Cortical AM281 133.2 ± 14.98% n=10). We favour the hypothesis that, after CB1R blockage, thalamic inputs start to present a stronger “synaptic tag” that marks them to capture more PRPs. Since the synthesis of PRPs is only triggered by the strong cortical stimulation, the available amount is not adequate for all three activated pools of synapses, resulting in transient forms of LTP, that decay do baseline values, in all inputs.

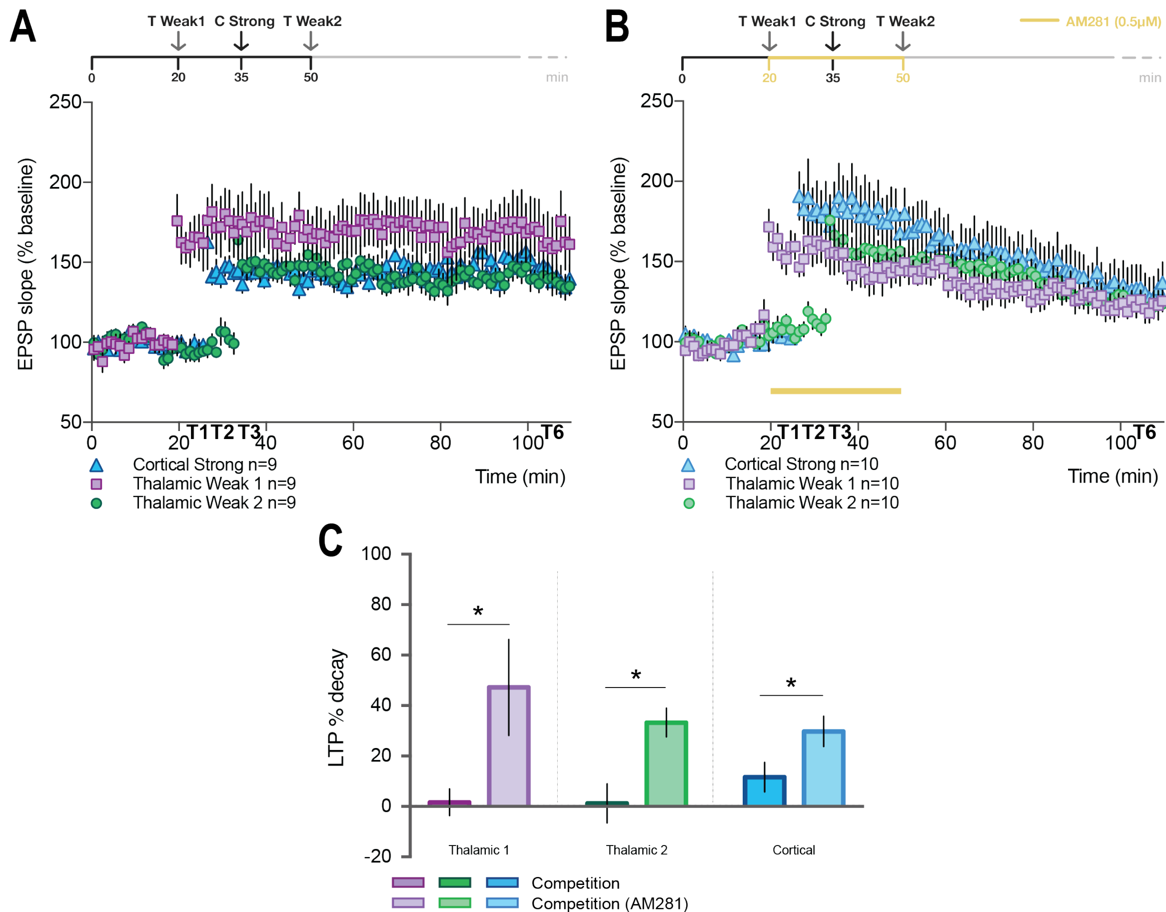


Figure 16: Synaptic competition between thalamic and cortical inputs into LA depends on the availability of plasticity-related proteins. (A) Induction of a maintained form of LTP in the cortical projection (△), by strong cortical tetanic stimulation (C Strong) (LTP T6 Cortical Competition 3 147.2 ± 8.02% n=9), is able to convert transient forms of LTP into maintained LTP in thalamic inputs stimulated 7.5-min before ( ) and 7.5-min after (○) C Strong (LTP T6 Thalamic 1 Competition 3 166.9 ± 19.34% n=9; Thalamic 2 Competition 3 139.5 ± 6.27% n=9). **(B)** AM281 application (0.5 μM) blocked LTP maintenance in all three inputs throughout the recording (LTP T6 Thalamic 1 AM281 123.3 ± 5.59% n=10; Thalamic 2 AM281 124.4 ± 5.18% n=10; Cortical AM281 133.2 ± 14.98% n=10). **(C)** Summary plots showing the percentage decay for the time window $(T_{initial}-T_{final})/T_{initial}*100$ for the conditions tested. AM281 application significantly increase LTP decay in thalamic and cortical inputs. (Kruskal-Wallis test was performed; the only data set that did not show a normal distribution was: Cortical pathway in B; *p-value ≤ 0.05). n=number of slices.

The statistical analysis of LTP decay [**Figure 15C**], shows that inhibition of CB1 receptors by AM281 application significantly increase LTP decay in thalamic and cortical inputs in the Competition 3 Protocol (LTP % decay Thalamic 1 Competition 3 1.7 ± 5.33% n=9, Thalamic 1 AM281 47.2 ± 19.04% n=10, H(1,19)=5.23 *p*=0.0222; ; T2 Thalamic 2

Competition 3 1.3 ± 7.82% n=9, Thalamic 2 AM281 33.3 ± 5.75% n=10, $F(1,19)=5.23$ $p=0.0222$; Cortical Competition 3 11.7 ± 5.93% n=9, Cortical AM281 29.8 ± 6.07% n=10, $H(1,19)=6.4067$ $p=0.0114$).

CHAPTER 5

DISCUSSION

With the present work, we addressed a form of heterosynaptic plasticity between thalamic and cortical projections to the LA, a well-known circuitry for its contribution to the formation of fear-conditioning memories. Using a protocol of 100-Hz tetanic stimulation, which we believe that resembles the activation of cortical and thalamic inputs during fear-learning, we induced transient and persistent forms of LTP (traditionally so-called by early-phase and late-phase LTP, respectively). Since whole-cell current-clamp recordings were recorded for at least 110-min, it allowed us to further understand and asses the mechanisms implicated in the maintenance of LTP.

Upon weak tetanic stimulation of the thalamic input or the cortical input we were able to induce transient forms of LTP that decayed to baseline values during the time of the recording. These results were consistent with the ones described previously in the Hippocampus (Fonseca et al., 2004) and in the LA (Fonseca, 2013).

Activation of both cortical and thalamic inputs into the LA is required in associative fear learning, as well as their interaction (Doyere et al., 2003). Interestingly, associated forms of plasticity, involving coincident activation of thalamic and cortical inputs had already been described (Humeau et al., 2003). Since we wanted to mimic what happens in associative fear learning (wherein cortical and thalamic information reach the LA and are associated), we decided to assess whether this co-stimulation of thalamic and cortical inputs would induce persistent forms of LTP. By concurrent stimulation of the thalamic and cortical inputs with a weak tetanus, we induced transient forms of LTP. This result can mean that LTP association after a weak co-stimulation of thalamic and cortical inputs is not sufficient to induce PRPs synthesis, which is essential for the induction and maintenance of long-lasting forms of LTP (Fonseca et al., 2006). Persistent forms of LTP at thalamic inputs require both pre and postsynaptic components (Fonseca, 2013), whereas persistent forms of LTP at cortical synapses are only expressed postsynaptically within our stimulation conditions. Thus, we may assume that this co-stimulation of thalamic and cortical inputs may not be sufficient to elicit presynaptic forms of LTP, required for the maintenance of LTP.

Next, we demonstrated a distinct form of associative plasticity at the LA, in which a transient form of thalamic LTP is converted into a maintained form of LTP, by capturing available PRPs synthesized upon the induction of a persistent form of LTP at cortical inputs. Prior results from our group, which showed that thalamic and cortical synapses engage in cooperation, were similar, however the order of the stimulations was different. Specifically,

in this previous study (Fonseca, 2013), a transient form of LTP was induced first, which allowed these synapses to be tagged, and then, within time intervals ranging from 7.5-min to 30-min, a persistent form of LTP was induced, leading to the synthesis of PRPs. The mechanism of synaptic cooperation, in which weak stimulated synapses behaved as persistent forms of LTP, was due to the duration of the tag which allowed previously activated synapses to capture PRPs that were only synthesized minutes later. However, we decided to opt for a strong-before-weak configuration so that we would not be limited by the duration of the tag, and later we could study the performance of these LTP forms after stimulating a new input with different time intervals. Thus, first we had to test whether in this configuration stimulated synapses engage in cooperation. We stimulated a cortical input with a strong tetanus, and 15-min later we stimulated a thalamic input with a weak tetanus. We found that a transient form of LTP, induced after a weak thalamic stimulation, is converted into a persistent form of LTP if a previous strong cortical stimulation occurs within this time interval. In other words, cortical and thalamic synapses cooperate by sharing the available PRPs synthesized upon cortical stimulation. Interestingly, by applying rapamycin, a protein synthesis inhibitor, during 40-min and starting 10-min before the cortical strong stimulation, we blocked synaptic cooperation. This result was consistent with the previous knowledge that the cortical-to-thalamic cooperation depends on *de novo* protein synthesis, as well as sharing and capture of PRPs (Redondo and Morris, 2011).

These experiments support the concept that plasticity factors, induced upon the stimulation of one input, are shared with other group of synapses after their subsequent activation (Frey and Morris, 1997). However, what does occur when the availability of these PRPs is reduced or limited? We found that thalamic and cortical synapses also compete. By stimulating a second thalamic input with a weak tetanus, 7.5-min after the first thalamic input, we increased the pool of activated synapses to which PRPs are allocated and triggered an imbalance in their distribution. Interestingly, we observed that LTP at both thalamic inputs decayed to baseline values, yet cortical LTP was maintained during the recording. Our interpretation is that generated PRPs are insufficient to stabilize all tagged synapses. Thus, the cortical input prevails to the detriment of the thalamic inputs, since it has been stimulated with a strong tetanus, presenting a stronger tag [**Figure 17**]. This is also in accordance with the concept that under certain conditions, STC depends on the strength of the tag as well as the amount of the different PRPs availability (Sajikumar and Korte, 2011).

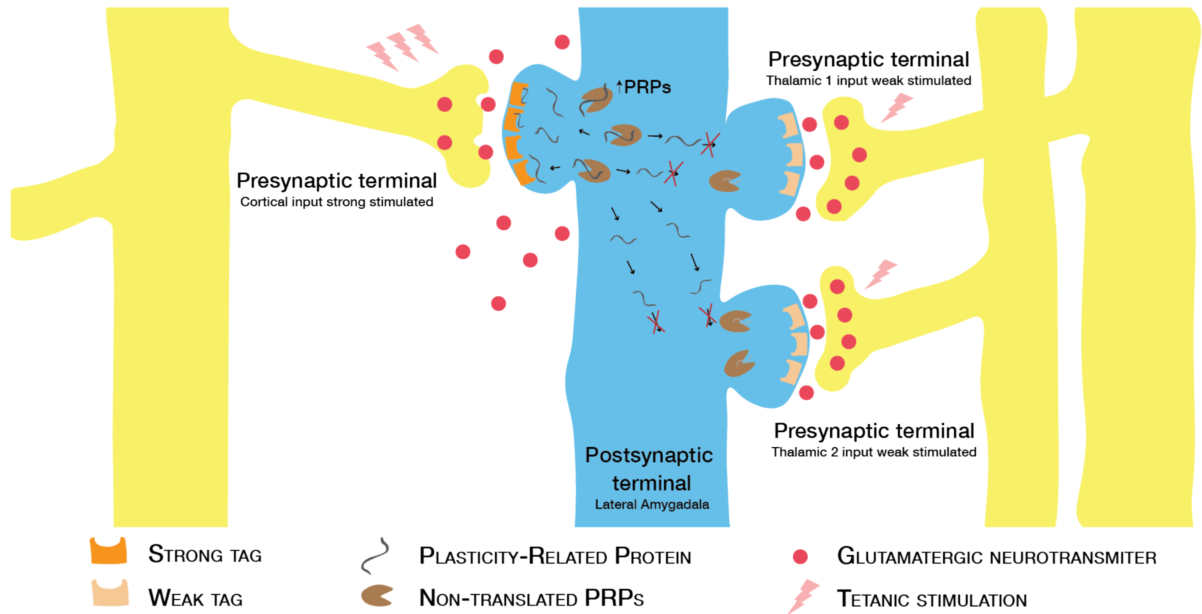


Figure 17: Potential mechanism for synaptic competition. Strong tetanic stimulation of the cortical input induces a persistent form of LTP that induces an upregulation of PRPs. Weak tetanic stimulations of the thalamic inputs are then induced (15-min and 22.5-min after the cortical stimulation). PRPs are distributed among activated synapses (cortical and thalamic). Since the amount of PRPs is limited, it is not possible to allocate equally at all tagged synapses. Thus, PRPs preferentially allocate at synapses with the stronger tag (cortical input).

Since this second thalamic input was induced 22.5-min after the cortical stimulation, we assessed whether within this time interval cortical and thalamic synapses still engage in synaptic cooperation. Persistent forms of LTP were induced at both inputs, suggesting synaptic tagging and capture of PRPs.

Previous work showed that CB1R activation is involved in the suppression of presynaptic thalamic LTP, upon the induction of postsynaptic thalamic LTP (Fonseca, 2013; R. M. Shin et al., 2010). Curiously, inhibition of CB1 receptors extends the time-window for cortical-to-thalamic cooperation, by increasing the duration of the tag and presumably its strength. We found that eCB signalling also modulates synaptic competition between cortical and thalamic synapses. When CB1R are blocked, by bath applying AM281, persistent forms of cortical and thalamic LTP are prevented, whereas upon activation of CB1R, by bath applying URB596, we observed long-lasting forms of LTP at all three groups of synapses. Our interpretation is that by inhibiting CB1R, thalamic inputs become more excitable, by

expressing both pre and postsynaptic forms of LTP. Then, this strengthens the tag and thalamic synapses begin to require greater amounts of PRPs, and competition. Thus, since PRPs are only synthesized upon strong cortical tetanic stimulation, the available amount is not sufficient to induce maintained forms of LTP at all groups of synapses (with a stronger tag) [**Figure 18B**]. On the other hand, by increasing the availability of eCB that bind presynaptically to CB1R, we propose that this leads to the stabilization of the first group of thalamic synapses weak stimulated [**Figure 18C**]. Therefore, the cortical and the second thalamic group of synapses have larger amounts of PRPs available, allowing their maintenance. Nevertheless, it is also important to point out that CB1R activation in normal conditions may also contribute to synaptic competition, since it inhibits presynaptic forms of LTP [**Figure 18A**].

To better characterize the time-related constraints of synaptic cooperation and competition mechanisms, we extended the time intervals between stimulations. We found that cortical and thalamic synapses do not engage in cooperation if the weak thalamic stimulation occurs 45-min after the strong cortical stimulation. Thus, we favour the hypothesis that at the moment when thalamic synapses are activated, they no longer have enough PRPs available so that maintained forms of LTP can be induced. Moreover, we extended the time-interval between the two thalamic stimulation up to 30-min, wherein the second thalamic input also has a 45-min interval relatively to the cortical pathway. We found that the first thalamic and the cortical inputs cooperate, whereas the second thalamic input failed to persist. We believe that the first two groups of stimulated synapses (cortical and the first thalamic) had sufficient PRPs to induce stable forms of LTP, whereas the second pool of thalamic synapses did not. By blocking CB1R, synaptic competition was triggered. Since transient forms of LTP were induced in all stimulated inputs, by considering our hypothesis that blocking CB1R favours the strengthening of the tag, this indicates that at the moment when the second thalamic input is stimulated, the first two inputs are not yet stabilized. Interestingly, we found that AM281 application did not result in an increase of PPF ratio at both thalamic and cortical inputs, which suggests that CB1R inhibition does not alter the probability of neurotransmitter release. Yet, it remains to address whether this drug affects upon LTP induction, specifically in the thalamic input, and therefore affects its presynaptic expression mechanism, contributing to the induction of maintained forms of LTP.

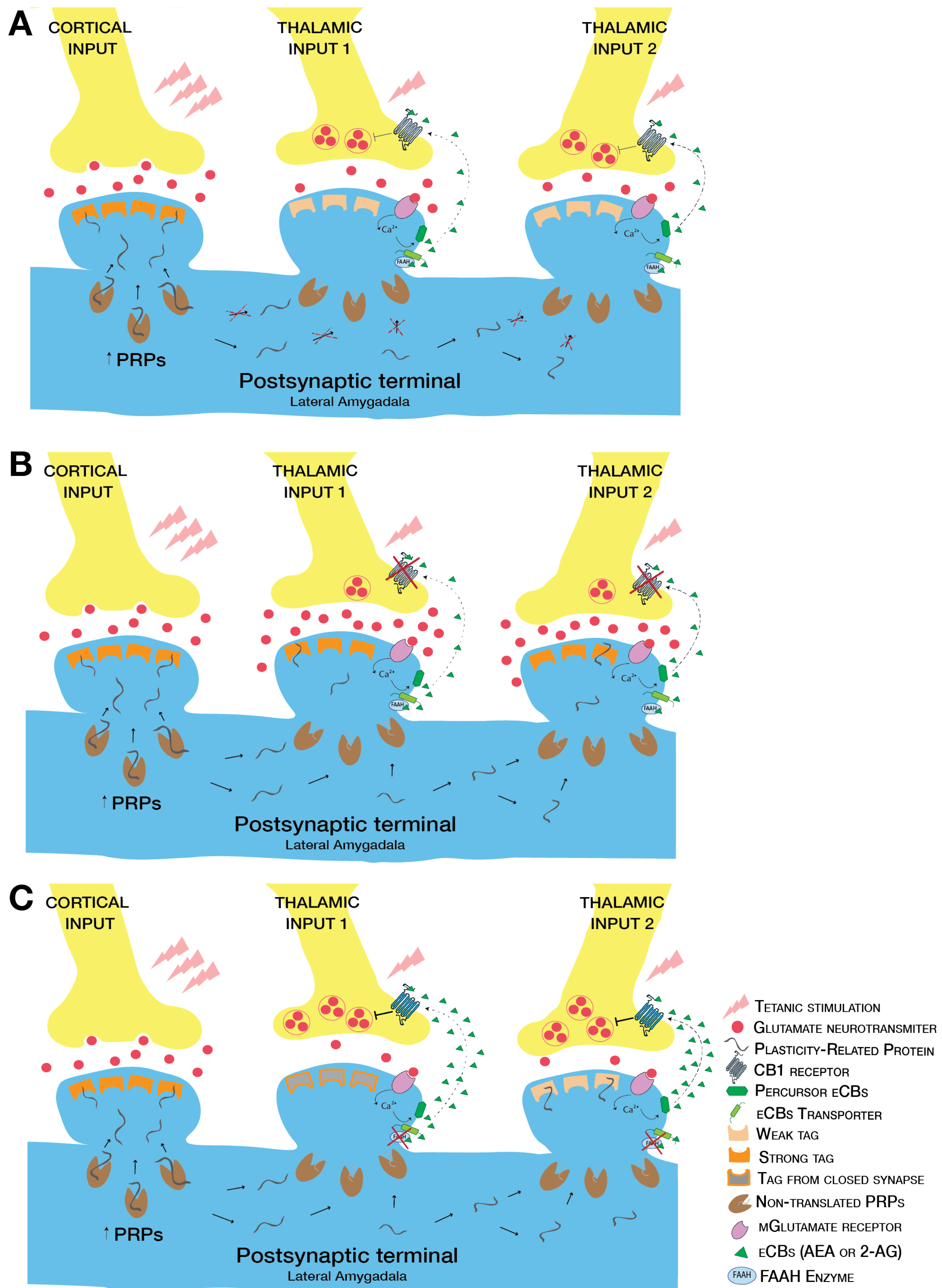


Figure 18: Simplified schematic diagram for the potential role of eCB signalling in synaptic competition. (A) Strong tetanic stimulation of the cortical input induces an upregulation of PRPs that are distributed among tagged synapses. PRPs tend to allocate at cortical synapses (with the stronger

tag). Since activation of CB1R by retrograde signalling contributes to the blockage of presynaptic forms of LTP upon weak thalamic stimulation, this mechanism may also contribute to the induction of transient forms of LTP at thalamic synapses (with weaker tag). **(B)** CB1R blockade leads to the release of more glutamate vesicles by the thalamic (presynaptic) neurons. This favours the tag to be stronger at thalamic activated synapses. Since PRPs upregulation only results from the strong cortical stimulation, and cortical and thalamic synapses now have stronger tags, PRPs tend to allocate equally at tagged synapses. Since the amount of PRPs is limited, this leads to the induction of transient forms of LTP at all three inputs. **(C)** Inhibiting FAAH enzyme results in increased availability of eCB that bind to CB1R. Activation of presynaptic CB1R enables thalamic input 1 to be more closed, therefore more stable, resulting in persistent forms of LTP. The second thalamic and the cortical inputs can now take advantage of the available PRPs and thus becoming a maintained form of LTP.

We decided to test whether synaptic competition between cortical and thalamic synapses also occurs if the order of the stimuli were different. We knew that thalamic synapses can capture PRPs if the interval until the strong cortical stimulation is up to 7.5-min (Fonseca, 2013). Therefore, we stimulated with a weak tetanus two thalamic inputs, one 7.5-min before the strong cortical stimulation, and the other 7.5-min after. We found that cooperation between the three group of activated synapses occurs, rather than competition. Since persistent forms of LTP were induced, we can assume that the amount of PRPs available was enough for their maintenance. Once again blocking CB1R resulted in transient forms of LTP, favouring the idea that thalamic synapses become marked with a stronger tag, making available PRPs insufficient to induce persistent forms of LTP in all inputs.

Taken together our results reveal that synaptic cooperation and competition between cortical and thalamic inputs into the LA is dependent on the amount of PRPs. In other words, synaptic competition can take off when protein resources are limited, as these plasticity factors are shared between activated synapses. Several studies have supported the idea that PRPs are encoded by specific genes, known as immediate-early genes (IEG), such as *egr*-1, c-*fos*, *Arc* and *Homer1a* (Gold, 2008; Minatohara et al., 2015; Rashid et al., 2016; Redondo and Morris, 2011). Intriguingly, these stimuli-induced genes have a rapid and transient responsiveness to synaptic activation (Guzowski, 2002; Okuno, 2011) and they are transiently expressed at activated neurons during fear-conditioning learning (Mamiya et al., 2009; Rosen et al., 1998). We may assume that in our experimental conditions, PRPs are transcribed within minutes after the strong cortical stimulation, reaching a maximum transcription threshold that tends to decrease over time. Thus, we propose that this transient and limited increase of PRPs, upon strong tetanic stimulation, can then explain the different time windows of cooperation and competition that we observed [**Figure 19**].

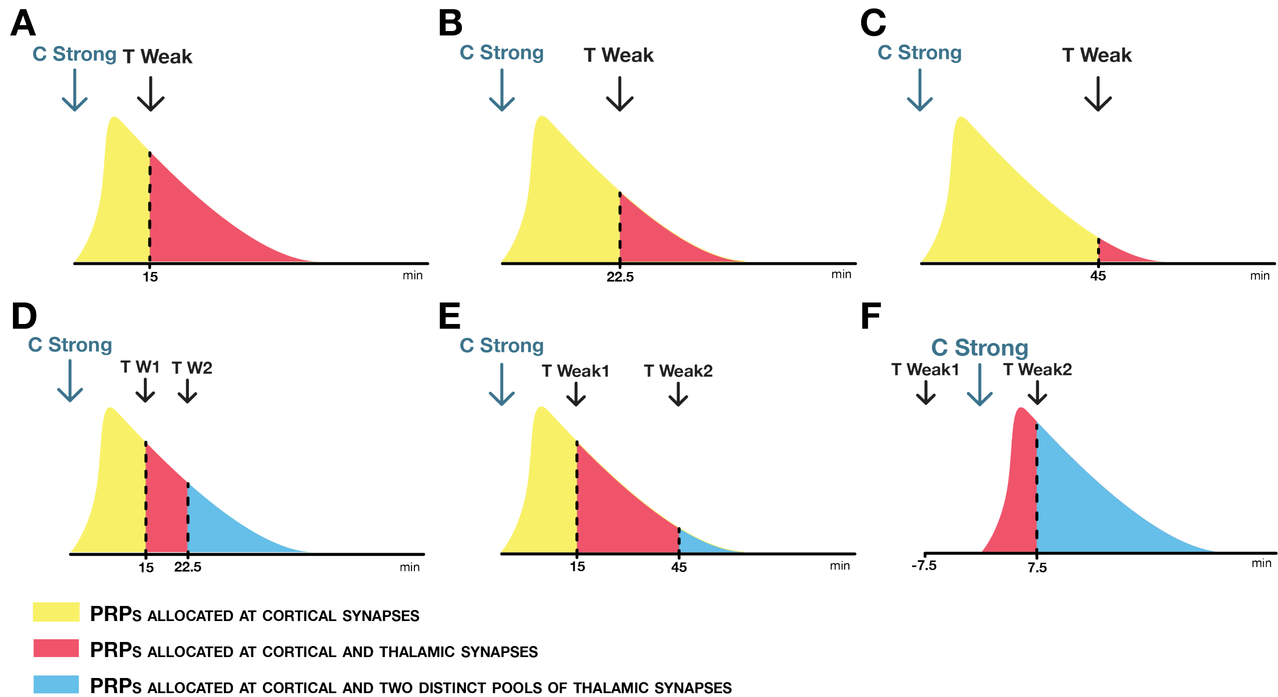


Figure 19: Schematic representation of the amount of PRPs available in our stimulation protocols. (A) Strong cortical stimulation (C Strong) is the trigger for PRPs synthesis. After 15-min a weak thalamic (T Weak) input is stimulated. The amount available of synthesized PRPs is distributed along activated synapses, leading to persistent forms of LTP at cortical and thalamic inputs, thus synaptic cooperation occurs. **(B)** A weak thalamic (T Weak) input is stimulated 22.5-min after stimulating the cortical input with a strong tetanic stimulation (C Strong). At the time that the thalamic synapses become tagged, the available amount of PRPs is distributed along activated synapses, leading to synaptic cooperation, and thus persistent forms of LTP at cortical and thalamic inputs. **(C)** If the thalamic input is stimulated with a weak tetanus (T Weak) 45-min after the strong cortical stimulation (C Strong), synaptic cooperation does not occur. At the time when the thalamic input is stimulated, the amount of PRPs available to be captured by activated thalamic synapses is not sufficient to induce a persistent form of LTP at thalamic synapses. **(D)** When a second thalamic input (T W2) is weak stimulated 7.5-min after the first weak thalamic input (T W1) synaptic competition occurs. The available amount of PRPs is distributed along activated synapses, but since T W1 and T W2 synapses do not have sufficient PRPs to induce persistent forms of LTP, or in other words to consolidate, both thalamic behave as transient forms of LTP. **(E)** Both cortical input (C Strong) and thalamic 1 input (T Weak1) when stimulated induce maintained forms of LTP since PRPs are allocated at those activated synapses. However, when we stimulate a second thalamic input (T Weak2) 30-min after T Weak1, at that time the amount of PRPs available is low to induce a persistent form of LTP at those synapses. Then, although C Strong and T Weak1 synapses have enough available PRPs to consolidate, as the T Weak2 synapses do not have them available, T Weak2 input behave as a transient form of LTP. **(F)** The first and the second thalamic (T Weak1 and T Weak2) inputs are stimulated 7.5-min before and 7.5-min after, respectively, the cortical (C Strong) stimulation. Since C Strong stimulation is the trigger for PRPs synthesis, the duration of the “synaptic tag” determines the ability of T Weak1 synapses to capture PRPs. At the time when T Weak2 stimulation is induced, although the available amount of PRPs is being distributed along T Weak1 and C Strong synapses, the amount of PRPs is higher is this situation, thus persistent forms of LTP are induced at all three stimulated pathways.

Once GABAergic activity may play a role in synaptic competition between cortical and thalamic inputs to the LA, we tested whether $GABA_A$ receptors inhibition, by aplying Picrotoxin, could extend the time window for cooperation. We found that blocking $GABA_A$ receptors indeed enhances cooperation, since both thalamic inputs (stimulated 15-min and 45-min after the cortical input stimulation) were converted into persistent forms of LTP. Interestingly, since the inhibitory gating of LTP at the thalamic afferents to the LA is controlled predominantly postsynaptic via $GABA_A$ receptors, and at cortical afferents this control is presynaptic via $GABA_B$ receptors (Ehrlich et al., 2009), we can deduce that Picrotoxin application affects mainly the induction of LTP at thalamic synapses. We verified that this enhancement of synaptic cooperation is also protein-synthesis dependent, since co-application of rapamycin and picrotoxin led to the induction of a transient form of LTP at the second thalamic input. This discovery is consistent with other works, wherein blocking inhibitory GABAergic transmission with a $GABA_A$ receptor antagonist affects protein expression pattern in the postsynaptic density (Ehlers, 2003). Thus, assuming that this inhibitory action decreases PRPs synthesis, under our conditions by blocking the GABAergic effect, increased levels of proteins become available, favoring synaptic cooperation [**Figure 20**].

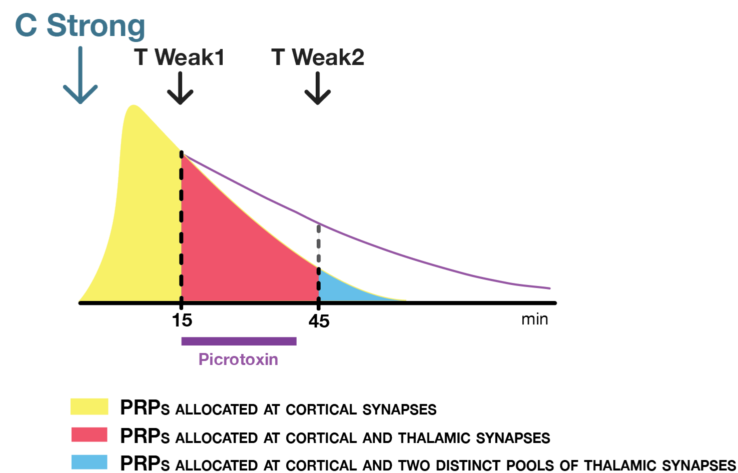


Figure 20: Schematic representation of the amount of PRPs available when Picrotoxin is applied. When Picrotoxin is applied, the inhibitory effect of $GABA_A$ receptors is blocked. We propose that the blockade of this inhibitory mechanism prevents synaptic competition, by increasing the amount of PRPs available. Thus, cortical inputs (C Strong) engage in synaptic cooperation with thalamic inputs.

Together, our results show that synaptic tagging and capture of PRPs is an universal mechanism for the association of events that occur separated by distant temporal windows (Redondo and Morris, 2011). Additionally, this supports the existence of a synaptic selection mechanism based on the competitive allocation of PRPs at activated synapses, wherein eCB signalling and GABAergic activity likely play an important part.

Given the similarity between cellular models of synaptic plasticity and memory formation, it is extremely important to explore how these processes of synaptic tagging, cooperation and competition have an impact on learning, such as discriminative fear learning. Curiously, during discriminative learning, when an animal learns to discriminate a CS^+/US from an CS^-/US association, LA pyramidal neurons increase their activity to the CS^+ and show a parallel decrease to the CS^- (Ghosh and Chattarji, 2015). Assuming that synaptic plasticity mechanisms are involved in the activity of the thalamic and cortical synapses, one hypothesis is that the increase in activity in CS^+ neurons is due to synaptic cooperation between thalamic and cortical inputs. Additionally, the decrease in activity in CS^- neurons is due to a competitive interaction between these two inputs, leading to an increase of the synaptic evoked response to the CS^+ as well as a decrement in CS^- evoked responses. Consistent with this, increasing PRPs availability decrease discriminative learning (Govindarajan et al., 2011; Han et al., 2008).

We can conclude that our observations have a major impact on the conceptual structure of associative and discriminative forms of fear learning, as they provide a cellular mechanism for the continuous integration of information into LA synapses. This project allowed us to further discern the synaptic rules underlying synaptic plasticity in the amygdala synapses, and provide us with valuable information to understand heterosynaptic plasticity between thalamic and cortical projections to the LA. Thus, by working in a very well-characterized area of the brain from the behavioural point of view, this work offers the possibility of integrating information from different levels of research, leading to a unifying vision of memory formation.

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