

UNIVERSIDADE DE LISBOA

Faculdade de Medicina da Universidade de Lisboa



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Mariana Martins Vieira Palmeiro Nunes

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

Coorientação: Prof. Doutora Luísa Maria Vaqueiro Lopes

Dissertação especialmente elaborada para a obtenção do grau de Mestre em Neurociências

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Resumo

A memória refere-se ao armazenamento de informação previamente aprendida. Esta capacidade permite que os animais ajustem e adaptem o seu comportamento ao ambiente, de acordo com aquilo que experienciaram. Assim, um dos mais fascinantes e centrais desafios da ciência moderna é a compreensão dos mecanismos neuronais que estão na base da aquisição e armazenamento de informação. Atualmente, pensa-se que a plasticidade sináptica esteja envolvida numa grande variedade de funções cerebrais, incluindo aprendizagem e memória. Em particular, a investigação desenvolvida tem-se concentrado em plasticidade sináptica dependente de atividade neuronal, como é o exemplo da potenciação de longa duração (LTP, do inglês *long-term potentiation*) ou da depressão de longa-duração (LTD, do inglês *long-term depression*) enquanto modelos celulares que estão na base da memória. Modelos clássicos de manutenção de LTP distinguem, pelo menos, duas fases: uma fase inicial (E-LTP, do inglês *early long-term potentiation*), independente de síntese proteica, que persiste durante minutos e uma fase tardia (L-LTP, do inglês *late long-term potentiation*), dependente de síntese proteica.

A indução de LTP é específica em relação ao *input*, ou seja, apenas as sinapses ativadas são potenciadas. Isto implica que macromoléculas necessárias para a manutenção de LTP, PRPs (do inglês *plasticity-related proteins*), devem ser, de alguma forma, recrutadas para sinapses ativadas. A hipótese de *tagging* e captura sináptica (STC, do inglês *Synaptic Tagging and Capture*), introduzida por Frey e Morris em 1997, propôs um mecanismo celular que concilia a especificidade de *input* da plasticidade sináptica com a alocação de PRPs. O modelo STC propõe que a atividade neuronal leva à formação de uma *tag* nas sinapses ativadas. A *tag*, temporalmente e espacialmente limitada, permite a essas sinapses a captura de moléculas necessárias à manutenção da plasticidade sináptica. Assim, a indução de LTP através de tetanização forte tem como consequência dois eventos dissociáveis: o estabelecimento local de uma *tag* sináptica e a síntese de PRPs. A indução de LTP através de tetanização fraca não leva à síntese de PRPs; no entanto, leva ao estabelecimento de uma “*tag*”. De uma forma geral, é a interação entre a *tag* e as PRPs que permite a manutenção da plasticidade sináptica. Neste contexto, podem surgir formas cooperativas de plasticidade sináptica, como observado em experiências anteriores: formas transientes de plasticidade, induzidas por uma estimulação fraca das sinapses, podem ser convertidas em formas persistentes através da utilização de PRPs sintetizadas devido à estimulação forte recebida por outro grupo de sinapses. A competição sináptica é outra possibilidade, em

situações de menor disponibilidade de PRPs ou maior número de *tags* ativas, por exemplo.

Embora não sejam ainda totalmente compreendidas, as vias de transdução de sinal responsáveis pelo estabelecimento da *tag* começam a ser elucidadas. De acordo com aquilo que se sabe atualmente, a *tag* dificilmente será equivalente a uma molécula (ou a um pequeno conjunto de moléculas). Em vez disso, a *tag* deve ser vista enquanto uma alteração local e transiente do estado da sinapse que, muito provavelmente, envolve uma complexa rede de proteínas e interações. Resultados anteriores do nosso grupo de laboratório demonstraram que o citoesqueleto de actina desempenha um papel crucial na captura de PRPs tanto para LTP como para LTD, suportando a hipótese de que uma remodelação dos filamentos de actina (*F-actin*), dependente de atividade sináptica, torna a sinapse localmente e transientemente permissiva a modificações plásticas. A proteína quinase CaMKII (do inglês *Ca²⁺/calmodulin-dependent protein kinase II*) demonstrou ser necessária para mecanismos de captura sináptica. A indução de plasticidade sináptica leva à dissociação da subunidade β da CaMKII do citoesqueleto de actina, algo necessário para a remodelação da sinapse. Esta deslocação (de cerca de 1 minuto) gera um sinal local que pode estar na base da *tag* sináptica. Curiosamente, a CaMKII leva à ativação de Cdc42 (do inglês *cell division control protein 42 homolog*), uma proteína pertencente à família das Rho GTPases, que também desempenha um papel central na regulação do citoesqueleto de actina nas espinhas dendríticas. A cofilina é uma proteína de distribuição ubíqua, cuja atividade leva à despolimerização dos filamentos de actina. Sabe-se que a Cdc42 está acima de PAK (do inglês *p21-activated kinase*), que promove a inibição da cofilina de duas formas. Por um lado, PAK fosforila e ativa a quinase LIMK (*LIM-kinase*), que, por sua vez, fosforila cofilina; por outro, bloqueia a atividade da fosfatase SSH (do inglês *Slingshot homologue*), inibindo assim a desfosforilação da cofilina e, conseqüentemente, a sua atividade. A Cdc42 é também responsável pela interação com a via N-WASP que leva à ativação do complexo Arp2/3, conhecido pelo seu papel na polimerização do citoesqueleto de actina. Foi previamente demonstrado que, ao contrário de outras Rho GTPases, a ativação da Cdc42 é restrita às espinhas dendríticas estimuladas (ou seja, demonstra especificidade de *input*), persiste durante mais de 30 minutos e depende da sinalização de BDNF (do inglês *brain-derived neurotrophic factor*).

Aqui, colocamos a hipótese de que a Cdc42 desempenha um papel essencial na persistência da plasticidade sináptica, sendo necessária para o estabelecimento de uma *tag* sináptica. Para testar esta hipótese, administrámos ML141, um inibidor farmacológico reversível e altamente seletivo para a Cdc42, em fatias hipocâmpais de

ratos juvenis em simultâneo com o registo eletrofisiológico de fEPSPs (do inglês *field excitatory post-synaptic potentials*) no *stratum radiatum* da área CA1.

Em condições de controlo, uma tetanização forte ao nível das colaterais de Schaffer leva à indução de uma forma persistente de LTP. Contudo, demonstrámos que, se o mesmo tipo de tetanização coincidir temporalmente com o período de aplicação do inibidor de Cdc42, a potenciação decai rapidamente para valores de *baseline*, como se observa em formas transientes de LTP. Estes resultados sugerem que a indução de formas persistentes de LTP depende da atividade de Cdc42.

Para além de se ter revelado enquanto necessária para a indução de LTP persistente, a Cdc42 revelou também ser necessária para a sua manutenção de acordo com uma janela temporal limitada. Ou seja, quando a aplicação do inibidor de Cdc42 é feita 40 a 70 minutos depois da indução de LTP, podemos observar um decaimento da potenciação sináptica. No entanto, a destabilização da manutenção de LTP persistente deixa de se verificar quando a aplicação do inibidor é feita 70 a 100 minutos depois da indução de plasticidade sináptica.

Finalmente, foram efetuadas experiências de cooperação sináptica. Primeiro, uma via é estimulada com tetanização fraca; passados 30 minutos, uma segunda via independente é estimulada com tetanização forte, que induz uma forma persistente de plasticidade. Em condições de controlo, a via que recebeu a estimulação fraca – que, por si só, levaria à expressão de uma forma transiente de LTP – é capaz de converter o seu LTP numa forma de LTP mais estável e persistente devido à estimulação forte na outra via. No entanto, quando a atividade de Cdc42 é inibida entre as duas estimulações, apenas a via que é estimulada com tetanização forte expressa uma forma persistente de LTP. Estes resultados sugerem que a inibição de Cdc42 interferiu com mecanismos de *tagging* e captura de macromoléculas necessárias à manutenção da plasticidade sináptica.

De uma forma geral, os nossos resultados apoiam a hipótese de que a Cdc42, ao regular o citoesqueleto de actina, interfere com a plasticidade sináptica, desempenhando um papel crucial na mesma. Estas e outras observações relativas aos mecanismos através dos quais o citoesqueleto é remodelado em consequência de atividade neuronal podem ter profundas implicações na compreensão dos mecanismos que estão subjacentes aos processos de memória e de aprendizagem. Para além disso, podem fornecer importantes alvos terapêuticos para doenças neuropsiquiátricas (como doença de Alzheimer e esquizofrenia, por exemplo) em que foram já identificadas

disfunções relacionadas com a actina ou com a complexa rede dos seus reguladores e interações que estabelecem entre si.

Keywords: Cdc42, Rho GTPases, LTP, STC, actina, plasticidade sináptica

Abstract

Maintained forms of Long-term potentiation (LTP) require de novo protein synthesis of plasticity-related proteins (PRPs). Since LTP is input-specific, it was proposed that activated synapses are tagged so that synthesized proteins are captured at modified synapses (synaptic tagging and capture hypothesis - STC). Although the nature of the synaptic tag remains unclear, it is generally accepted that it must be a local and transient molecular alteration caused by synaptic activation which can capture PRPs. Several molecules have been implicated in LTP maintenance by synaptic tagging and capture mechanisms, namely CaMKII, PKA and BDNF. Previous results from our laboratory group have shown a critical role of actin dynamics in the tagging and capture of PRPs in both LTP and LTD, supporting the hypothesis that an activity-dependent remodeling of F-actin through CaMKII activation, renders the synapse locally and transiently permissive to plasticity modifications. Interestingly, CaMKII leads to activation of Cdc42, a Rho GTPase that also plays a role in regulating the actin cytoskeleton in dendritic spines. Cdc42 is known to be upstream of PAK (p21-activated kinase), that phosphorylates and activates LIM-kinase (LIMK), which, in turn, phosphorylates cofilin, inhibiting its actin-depolymerizing activity. Cdc42 is also responsible for interacting with the WAVE1/N-WASP pathway to activate Arp2/3 complex-dependent actin polymerization. One hypothesis is that Cdc42 activity promotes actin polymerization. Since Cdc42 activity has been shown to be heavily restricted to the stimulated spine (input-specificity), last more than 30 minutes and is dependent on BDNF signaling, we hypothesize that Cdc42 plays a crucial role in the setting of the synaptic tag. Here, we assess the role of Cdc42 in synaptic tagging and LTP maintenance by pharmacologically inhibiting Cdc42 activity while performing electrophysiological recordings in rat hippocampal slices. We found that inhibition of Cdc42 does not interfere with the expression of a transient form of LTP but blocks the induction of a maintained form of LTP. Moreover, the inhibition of Cdc42 blocks the maintenance of synaptic plasticity within a limited time window. Finally, we show that Cdc42 inhibition interferes with synaptic tagging and capture mechanisms. Since Cdc42 activation promotes actin polymerization we propose that Cdc42 inhibition may interfere with the maintenance of LTP by promoting actin depolymerization. Our results suggest that, by interfering with the actin cytoskeleton, Cdc42 interferes with synaptic plasticity.

Keywords: Cdc42, Rho GTPases, LTP, STC, actin, synaptic plasticity

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List of Abbreviations

- ABPs** – actin binding proteins
- ACSF** – artificial cerebrospinal fluid
- ADP** – adenosine diphosphate
- AMPAR** - α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
- Arp2/3** – actin-related proteins 2/3
- ATP** – adenosine triphosphate
- BDNF** – brain-derived neurotrophic factor
- CA1** – cornu ammonis 1
- CA3** – cornu ammonis 3
- CaMKII** – Ca^{2+} /calmodulin-dependent protein kinase II
- Cdc42** – cell division control protein 42 homolog
- DMSO** - Dimethyl sulfoxide
- E-LTP** – early long-term potentiation
- fEPSPs** – field excitatory post-synaptic potentials
- GAPs** – GTPase activating proteins
- GDIs** – guanine nucleotide dissociation inhibitors
- GDP** – guanosine diphosphate
- GEFs** – guanine nucleotide exchange factor
- GTP** – guanosine triphosphate
- HFS** – high frequency stimulation
- IA** – inhibitory avoidance
- IRSp53** - insulin receptor tyrosine kinase substrate p53
- L-LTP** – late long-term potentiation
- LTD** – long-term depression
- PPF** – long-term memory
- LTP** – long-term potentiation
- mDIA2** – mammalian Diaphanous-related formin 2
- mRNA** – messenger ribonucleic acid
- NMDAR** - N-methyl-D-aspartate receptor
- N-WASP** – neural Wiskott-Aldrich syndrome protein
- PAK** – p21-activated kinase
- PKA** – protein kinase A
- PKC** – protein kinase C
- PPF** – paired pulse facilitation
- PRPs** – plasticity-related proteins

PSD – postsynaptic density
PTMs – post-translational modifications
Rac1 - Ras-related C3 botulinum toxin substrate 1
RhoA – ras homolog gene family, member *A*
ROCK – Rho-associated protein kinase
LIMK – LIM domain kinase
SEM – standard error of the mean
SPM – synaptic plasticity and memory
SSH – slingshot homolog
STC – synaptic tagging and capture
STDP – spike-timing-dependent plasticity

1. Introduction

Understanding how the brain acquires and stores information is one of the biggest and most fascinating challenges of science. Through the acquisition of knowledge about the world (learning) and the ability to maintain and reconstruct that knowledge (memory), an animal is able to adjust its behavior and adapt to its environment (Eric R. Kandel, Dudai, & Mayford, 2014). The brain mediates what the animal experiences through the conversion of sensory information into functional neuronal changes (E. R. Kandel, 2001). One can think of learning as a new memory formation process, in which a cascade of cellular events lead to structural and functional changes (Ramirez, 2018). But what exactly changes in the brain after learning? And how is the learned information maintained?

1.1. Cellular Basis of Learning and Memory

Changes in the strength of connections between neurons is widely assumed to be the cellular mechanism through which encoding and storage of information in the nervous system are possible (Mayford, Siegelbaum, & Kandel, 2012). These changes result in altered neuronal states, can persist for various periods of time and are broadly referred to as synaptic plasticity (Kukushkin & Carew, 2017). The Synaptic Plasticity and Memory Hypothesis (SPM) reflects on the possible relationship between synaptic plasticity and memory. In 2000, Martin and colleagues (Martin, Grimwood, & Morris, 2000) delineated the SPM hypothesis as follows:

“Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation and is both necessary and sufficient for the information underlying the type of memory mediated by the brain area in which that plasticity is observed.”

However, we must not make the oversimplification of assuming that synaptic plasticity equals memory. The SPM hypothesis asserts that activity-dependent plasticity is the fundamental mechanism responsible for creating and storing memory traces. In this sense, one should not confuse memory with the biological mechanism that allows it (Martin et al., 2000; Takeuchi, Duzkiewicz, & Morris, 2013). Although there is still a debate regarding whether synaptic plasticity is sufficient to explain memory encoding and storage (Neves, Cooke, & Bliss, 2008; Ryan, Roy, Pignatelli, Arons, & Tonegawa, 2015), the use of new technological approaches continue to provide evidence in support of the idea that one of the major mechanisms through which engrams are stored in the brain are changes in the strength of connections between neurons (Takeuchi et al., 2013).

1.1.1. Synaptic Plasticity in the Hippocampus

In 1949, Donald Hebb advanced a neurophysiological postulate of a widely distributed biological mechanism that could underlie the basis of information storage in the brain (Josselyn, Köhler, & Frankland, 2017), through the formation of new synaptic connections and the reorganization of existing ones (Tonegawa, Morrissey, & Kitamura, 2018). Hebb's renowned statement is included in his book (Hebb, 1949) as follows:

“When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.”

Only decades later would empirical evidence emerge to support Hebb's mechanistic insight. The extensively studied model of synaptic plasticity, long-term potentiation (LTP), was first described in the rodent hippocampus (T. V. P. Bliss & Lømo, 1973), where it was reported that brief tetanic stimulation could induce potentiated synaptic efficacy at perforant path-granule cell synapses, persisting for several hours after the tetanus. Subsequent studies found that LTP displayed many interesting properties (persistence, input-specificity, associativity and cooperativity) that made it an attractive cellular model of learning and memory (T. Bliss, Collingridge, & Morris, 2003; T. V. P. Bliss & Collingridge, 1993).

In 1986, a pharmacological study showed that blocking the N-methyl-D-aspartic acid (NMDA) receptors led to impairment in spatial learning and suppressed LTP *in vivo* (Morris, Anderson, Lynch, & Baudry, 1986). The most widely studied form of synaptic plasticity in the vertebrate brain is the NMDA receptor-dependent LTP (Tim V.P. Bliss, Collingridge, Morris, & Reymann, 2018). The properties of the NMDA receptor can explain the associative and activity-dependent properties of these forms of LTP (Eric R. Kandel et al., 2014). NMDA receptors are sensitive to magnesium ion concentrations in a voltage-dependent manner. When sufficient depolarization is provided, the magnesium block is alleviated and ion passage (upon binding of glutamate) may occur through the NMDAR (N-methyl-D-aspartate receptor) channel (Citri & Malenka, 2008). In order to become active, NMDA receptors require both pre-synaptic glutamate release and depolarization of the post-synaptic membrane and are thus said to be the “coincidence detector” for the input-specificity mechanisms (Collingridge, 2003). This form of LTP can be considered a Hebbian process, since it requires the coincident activity of both pre-synaptic and post-synaptic neurons (Neves et al., 2008).

NMDA receptors allow the entry of calcium into post-synaptic neuronal cells. (Robert C Malenka, Kauer, Zucker, & Nicoll, 1988). The postsynaptic calcium signal can trigger a wide range of intercellular signalling pathways (e.g. CaMKII, PKA, PKC) that have been implicated in several processes, such as LTP maintenance, cytoskeletal rearrangement or AMPA receptor insertion into the postsynaptic membrane (Herring & Nicoll, 2016; Hou, Gilbert, & Man, 2011; Huang et al., 2013; Sanhueza & Lisman, 2013).

While LTP is the most studied form of synaptic plasticity in the hippocampus, there are a variety of other plasticity mechanisms in the mammalian brain, such as LTD (long-term depression) or STDP (spike-timing-dependent plasticity) (Robert C Malenka & Bear, 2004; Song & Abbott, 2001). Moreover, LTP is not a unitary phenomenon and not all forms of LTP are NMDA receptor-dependent, as is the case of LTP at the mossy fiber synapse on CA3 neurons (Nicoll & Malenka, 1995).

The phenomenon of LTP has, since its discovery, been heavily associated with learning and memory (Lynch, 2004; R C Malenka & Nicoll, 1997) but only recently have researchers been able to provide a direct demonstration that hippocampal LTP is actually induced by learning. Inhibitory avoidance (IA) memory is rapidly acquired and dependent on the hippocampus. In 2006, Whitlock and colleagues provided electrophysiological evidence that IA training induced LTP-like potentiation in hippocampal Schaffer collateral-CA1 synapses (Whitlock, 2006). Hippocampal glutamate receptors were phosphorylated, AMPA receptor trafficking occurred after IA training and the acquisition of the avoidance response required NMDA receptor activation. Subsequent induction of LTP with HFS (high-frequency stimulation) was impaired only at sites where potentiation was observed during learning and the authors suggested that this could imply that IA and HFS may share mechanisms through which synaptic transmission in CA1 is increased. Since then, learning-induced enhancement in synaptic strength has been described in the CA3-CA1 synapses with other tasks that also engage the hippocampus, such as eyeblink conditioning (Gruart, 2006) and novel object recognition (Izquierdo, Delgado-Garcia, Cammarota, Clarke, & Gruart, 2010).

1.1.2. The Synaptic Tagging and Capture Model of LTP

The aging studies that Barnes performed, shortly after the discovery of LTP in the rodent brain, allowed her to raise the possibility that the temporal persistence of LTP might be one determinant (though not the only one) of the persistence of memory, or at the very least, synaptic plasticity and memory share common mechanisms for persistency (Barnes, 1979). Synaptic potentiation outlasts the events of its induction but,

in general, LTP decays back to baseline values within a few hours (Takeuchi et al., 2013). Classically, two phases of LTP are differentiated: an early, short-term phase (E-LTP), which lasts minutes, and a later, long-term phase (L-LTP), which lasts hours (Nguyen, Abel, & Kandel, 1994). The use of protein synthesis inhibitors, such as anisomycin, rendered experimental evidence suggesting that maintained forms of LTP required de novo protein synthesis (Uwe Frey, Krug, Reymann, & Matthies, 1988). Thus, L-LTP shares the requirement for de novo protein synthesis and mRNA with long-term memory (Barco, Lopez de Armentia, & Alarcon, 2008; Goelet, Castellucci, Schacher, & Kandel, 1986). In addition, one of the properties of LTP is its input-specificity (i.e. only activated synapses are potentiated). How, then, are the necessary proteins and mRNA selectively targeted to synapses during tetanization to ensure plasticity maintenance?

In 1997, Frey and Morris proposed a possible solution when they introduced the concept of synaptic tagging: activated synapses are “tagged” so that newly synthesized plasticity-related proteins (PRPs) can be specifically localized to these activated synapses allowing input-specific maintenance of plasticity (Uwe Frey & Morris, 1997, 1998). The authors used transverse hippocampal slices from adult rats and applied tetanic stimulation to two independent synaptic inputs in the CA1 region. It was shown that the induction of a long-lasting form of LTP in one set of synapses causes conversion of short-lasting LTP at another, independent set of synapses into a long-lasting form. The stabilization of the short-lasting form of LTP (induced by weak tetanic stimulation) is prevented if protein synthesis inhibitors are applied during the induction of the long-lasting form of LTP (induced by strong tetanic stimulation). These experiments paved the way for the Synaptic Tagging and Capture (STC) model (Barco et al., 2008). The STC model proposes that synaptic activity leads to two dissociable events: (1) a local “tag” setting and (2) the synthesis of plasticity related proteins (PRPs) (Roger L. Redondo & Morris, 2011). A molecular “tag” is left at synapses that are affected by the induction of either short-lasting or long-lasting forms of synaptic plasticity; the “tag” allows those synapses to “capture” the PRPs that were made available in response to induction of long-lasting plasticity. The persistence of LTP is achieved if both tags and PRPs are available within a specific time window and it is the tag-PRP interaction that leads to a maintained form of LTP (S. Frey & Frey, 2008; U. Frey & Morris, 1998). The earlier experiments made by Frey and Morris (Uwe Frey & Morris, 1997) were the first demonstration that synapses could cooperate by sharing PRPs, allowing neurons to integrate multiple events in a large window of time. Interestingly, synapses may also be involved in competitive interactions, and this would be most likely in circumstances where the availability of PRPs is limited (Fonseca, Nägerl, Morris, & Bonhoeffer, 2004).

The setting of the synaptic “tag” and the long-lasting maintenance of LTP are independent processes and can occur separately in time (R. L. Redondo et al., 2010; Sajikumar, Navakkode, & Frey, 2007; Wang, Redondo, & Morris, 2010). Although not fully understood, the signal transduction pathways responsible for setting the *tag* are beginning to be elucidated. It is currently accepted that the tag isn’t equal to one molecule in particular. Instead, the tag should be considered as a local, transient alteration of the synapse state that, most likely, involves a complex network of proteins and interactions (Roger L. Redondo & Morris, 2011).

1.2. The role of actin dynamics in synaptic plasticity

The actin network is essential for sculpting and maintaining cell shape and actin dynamics support a multitude of important biological processes (e.g. cell division, intracellular protein trafficking, cell motility) (Bosch et al., 2014; Hanley, 2014). Actin exists in two states in the cell: monomeric globular actin (G-actin) and as an asymmetric two-stranded helical filament (F-actin) that is formed by polymerization of monomeric G-actin. These two forms of actin undergo a cycle called treadmilling. ATP-bound G-actin is added to the fast-growing end (barbed end or plus end) and ADP-bound G-actin is dissociated from the other side (pointed end or minus end) of F-actin (Blanchoin, Boujemaa-Paterski, Sykes, & Plastino, 2014). The difference in polymerization rates between the two extremities result in a net turnover of the filaments. In spines, the cycle of treadmilling is fast and most actin monomers are replaced every minute (Nakahata & Yasuda, 2018).

In mature neurons, actin is the most prominent cytoskeletal protein at synapses. In particular, actin is highly enriched at dendritic spines, specialized compartments that mediate most of the excitatory transmission in the brain (Saneyoshi & Hayashi, 2012). Spines contain a postsynaptic density (PSD), a matrix of proteins located below the membrane that is in close interaction with actin filaments; the PSD includes receptors, channels and signalling molecules that couple synaptic activity with postsynaptic signaling (Lamprecht & LeDoux, 2004). The actin cytoskeleton controls the organization of the PSD, which is essential for the stability of LTP (Roger L. Redondo & Morris, 2011; Saneyoshi & Hayashi, 2012). Actin dynamics are essential for the structural modification of synapses and for spine-specific, long-term structural and functional synaptic plasticity. In some circumstances, the changes in synaptic efficacy are accompanied by structural plasticity. For example, upon LTP induction, existing spines are enlarged, an event that

is associated with an increase in the number of AMPA receptors at the synapse (Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004; Ken-Ichi Okamoto, Nagai, Miyawaki, & Hayashi, 2004). Previous results from our laboratory group have shown a critical role of actin dynamics in the induction and maintenance of both LTP and LTD (Fonseca, 2012; Szabó, Manguinhas, & Fonseca, 2016). Synaptic stimulation can trigger long-lasting remodeling of the actin network at both pre-and post-synaptic sites. (Colicos, Collins, Sailor, & Goda, 2001). Inside dendritic spines, actin is organized in dynamic pools of actin filaments (Honkura, Matsuzaki, Noguchi, Ellis-Davies, & Kasai, 2008). In spines at rest, the pool at the tip of the spine shows a very high turnover, particularly when compared to the base of the spine, that is much slower. In activated spines (by the release of caged glutamate), a third pool of F-actin is formed, associated with spine enlargement. The pool of F-actin associated with spine enlargement was relatively stable and necessary for the long-term increase in spine size in a CaMKII-dependent way (Honkura et al., 2008). Curiously, the increase in F-actin - associated with maintained forms of LTP (Fukazawa et al., 2003) - is preceded by a NMDAR-dependent, transient decrease in synaptic F-actin (Ouyang et al., 2005; Saneyoshi & Hayashi, 2012). This suggests that remodeling of F-actin in spines is promoted by early plasticity. In 2015, Kim and colleagues (K. Kim et al., 2015) demonstrated that CaMKII is involved in a “gating mechanism” through actin modulation. The authors found that CaMKII β is transiently dissociated from the actin filaments and that this is necessary for synapse remodeling and LTP induction. Upon CaMKII β dissociation, the actin cytoskeleton is permissive to modifications from actin regulators. CaMKII β quickly reassociates and stabilizes F-actin, within a short period of around 1 minute. Noteworthy, the time window during which CaMKII is displaced from the actin filaments (1 minute) in an activity-dependent manner is far smaller than the duration of the synaptic tag (30 minutes to 1 hour), as was previously assessed through weak-before-strong experiments in the Schaffer collateral pathway (Fonseca, 2012; Szabó et al., 2016).

A number of different proteins regulate actin behavior within a cell. Some actin-binding proteins (ABPs) can alter actin-filament dynamics. Actin remodeling is controlled, among other proteins, by cofilin, an ubiquitous actin-binding protein that is essential for depolymerizing actin filaments (Chen, Rex, Casale, Gall, & Lynch, 2007). Cofilin-mediated dynamics regulate spine morphology and AMPAR trafficking during synaptic plasticity (Gu et al., 2010). The Arp2/3 (Actin-related protein 2/3) complex activity also plays an important role in actin cytoskeleton reorganization since it mediates the nucleation of actin polymerization (Takenawa & Suetsugu, 2007). In response to synaptic

activity, the cellular functions of ABPs are exploited by signal transduction machineries to adapt synaptic morphology (Cingolani & Goda, 2008).

1.3. Rho GTPases – central regulators of actin organization

1.3.1. The Rho GTPases protein family

Rho GTPases and their downstream effectors are key regulators of the actin cytoskeleton in response to extracellular signals (Lamprecht & LeDoux, 2004; Saneyoshi, Fortin, & Soderling, 2010). As such, these proteins coordinate a wide range of actin-dependent cellular activity, from neuronal development processes to structural plasticity of dendritic spines (Heasman & Ridley, 2008; Luo, 2002). The protein family of Rho GTPases belong to the Ras superfamily of small GTPases and are highly conserved among all eukaryotic life. In mammals, the family comprises 20 members structured into 8 different subfamilies (Boureux, Vignal, Faure, & Fort, 2007).

Most Rho GTPases act as intracellular molecular switches that cycle between an active (GTP-bound) form and an inactive (GDP-bound) form. Three types of protein regulate this cycling: (1) guanine nucleotide exchange factors (GEFs), that catalyze the GDP-GTP change and lead to Rho GTPase activation; (2) GTPase activating proteins (GAPs), that enhance the intrinsic GTP hydrolysis rate of Rho GTPases, thereby leading to their inactivation; and (3) guanine nucleotide dissociation inhibitors (GDIs), that sequester the GDP-bound form of some GTPases in the cytosol and prevent them from being activated by GEFs. Rho GTPases are able to bind to their downstream effectors when they are in their active, GTP-bound conformation (Hall & Nobes, 2000; Heasman & Ridley, 2008). Although most functional and mechanistic data pertains to classical Rho GTPases (e.g. Rac1, RhoA and Cdc42), some Rho GTPases are said to be “atypical” (such as RhoU or RhoV). Atypical GTPases are not generally regulated by GTP-GDP cycling and therefore do not require GEFs and GAPs. Consistently, Rho GTPases can be regulated by other mechanisms at the level of gene expression (through epigenetics and miRNA regulation) and at the post-translational level (Hodge & Ridley, 2016). Post-translational modifications (PTMs), regulate several aspects of Rho GTPases’ signaling: prenylation and palmitoylation play a crucial role in determining the subcellular localization of Rho GTPases, phosphorylation and SUMOylation regulate GTPase activity and ubiquitylation is important for modulating Rho GTPase protein levels. Like Rho GTPases, several of their regulatory proteins (i.e. GEFs, GAPs and GDIs) can also

undergo post-translational modifications, creating a complex network of interactions to determine the precise spatiotemporal activation of Rho GTPases (Hodge & Ridley, 2016).

1.3.2. The Cdc42 signaling pathways in actin dynamics

The three best studied members of the Rho GTPase family are RhoA, Rac1 and Cdc42. All three members are found at glutamatergic synapses, where they have a marked effect on the morphology of dendritic spines (Herring & Nicoll, 2016). Cdc42 is thought to underlie the formation of filopodia, cytoplasmic extensions that contain parallel bundles of F-actin and thus depend on the polymerization of actin (Ridley, 2011). Several downstream effectors for Cdc42 have been implicated in actin dynamics. For instance, Cdc42 induces actin polymerization in a GTP-dependent manner by binding to N-WASP (neural Wiskott Aldrich syndrome protein), which activates the actin-related protein (Arp2/3) complex (Wegner et al., 2008). The knock down of N-WASP leads to significant reduction of the number of excitatory synapses (specifically the ones formed on spines) in hippocampal neurons. Cdc42 also activates IRSp53 (insulin-receptor substrate p53) to induce branched actin filaments, again using the Arp2/3 complex (Heasman & Ridley, 2008). Mammalian diaphanous (mDIA) proteins were found to stimulate the polymerization of unbranched actin filaments. These proteins are members of the formin family of ABPs. In particular, there is mDia2, a Cdc42 target that, like IRSp53, was shown to mediate filopodia formation (Peng, Wallar, Flanders, Swiatek, & Alberts, 2003). Finally, one of the best characterized downstream effectors for both Rac1 and Cdc42 is PAK (p21 activated kinase). Cofilin is a member of the actin depolymerization factors and can sever F-actin and promote depolymerization. The activity of cofilin is regulated by phosphorylation at amino acid residue serine 3 by LIM-Kinase (LIMK) and by dephosphorylation by Slingshot (SSH) (Bamburg & Bernstein, 2016). The phosphorylation of cofilin inhibits its activity and allows actin polymerization (Lamprecht & LeDoux, 2004). Thus, activation of Cdc42 activates Pak, which in turn activates LIMK, leading to the downregulation of cofilin activity and inhibition of actin depolymerization. Therefore, in general, the influence of Cdc42 over actin dynamics seems to be directed towards actin polymerization.

1.3.3. Rho GTPases and synaptic plasticity

The Rho family of GTPases have important roles in the morphogenesis of the dendritic spines and synaptic plasticity through the modulation of the actin cytoskeleton organization (Saneyoshi et al., 2010). In a recent set of studies, two-photon photolysis

of caged glutamate was used to assess the behavior of Rac1, Cdc42 and RhoA in individual spines upon glutamate uncaging and subsequent dendritic plasticity. For all three studied Rho GTPases, the time course of their activation was similar: rapid activation followed by persistent activation that lasted for more than 30 min (Hedrick et al., 2016; Murakoshi, Wang, & Yasuda, 2011). However, their activity patterns were shown to be different: Cdc42 activation was restricted to the stimulated spine (i.e. was input-specific) whilst RhoA and Rac1 diffused out of the stimulated spine and spread over a few micrometers along the dendrite (Hedrick et al., 2016; Murakoshi et al., 2011). The authors also showed that pharmacological inhibitors of Pak (downstream effector of Rac1 and Cdc42) and ROCK (downstream effector of RhoA) blocked the structural enlargement of dendritic spines associated with LTP induction (Murakoshi et al., 2011), suggesting that Rho GTPases play a crucial role in the plasticity of glutamatergic spines. This was consistent with previous studies that showed that mice lacking the genes coding for those protein effectors or expressing a dominant negative form of PAK displayed LTP impairment (Asrar et al., 2009; Hayashi et al., 2004; Zhou, Meng, Asrar, Todorovski, & Jia, 2009). During LTP induction, NMDA receptor activation results in the activity of calcium-mediated CaMKII, a protein that is known to be essential for synaptic plasticity, learning and synaptic organization (Hell, 2014). The activity of Rho GTPases depends on CaMKII activity (Herring & Nicoll, 2016). Moreover, it was shown that BDNF, a neurotrophin that is also critical for synaptic plasticity (Kowiański et al., 2018), is sufficient to activate Rac1 and Cdc42 (Hedrick et al., 2016). Overall, Rho GTPases have a central role in the regulation of the actin cytoskeleton in dendritic spines and, therefore, a central role in the plasticity of dendritic spines.

2. Rationale and Aims

2. Rationale and Aims

The Synaptic Tagging and Capture (STC) hypothesis presents a great working model to study the role of macromolecules in the induction and maintenance of LTP (R. Redondo & Morris, 2013). The stabilization of LTP requires the alteration of dendritic spine architecture and the reorganization of the PSD, both of which depend on the actin cytoskeleton (Roger L. Redondo & Morris, 2011; Saneyoshi & Hayashi, 2012). We have previously shown that the regulation of actin dynamics plays a critical role in the maintenance and synaptic capture of LTP (Fonseca, 2012) and LTD (Szabó et al., 2016). Thus, actin and its complex network of upstream regulators seem to be heavily involved in synaptic tagging and capture mechanisms. Cdc42 is known to be upstream of several pathways that regulate actin dynamics (through N-WASP or PAK, for example) (Heasman & Ridley, 2008). Moreover, Cdc42 activity displays input-specificity (active Cdc42 is heavily restricted to the stimulated spine), lasts more than 30 minutes and depends on CaMKII and BDNF signaling (Hedrick et al., 2016; Murakoshi et al., 2011) molecules that are implicated in synaptic tagging (Lu, Christian, & Lu, 2008; R. L. Redondo et al., 2010). Hence, it is quite plausible that Cdc42 plays an important part in the molecular alterations that underlie the synaptic tag.

The simple laminar pattern of neurons and neural pathways of the hippocampus enables the use of extracellular recording techniques to record synaptic events, making it a great experimental system for studies of synaptic plasticity (Andersen, Morris, Amaral, Bliss, & O'Keefe, 2007). Acute transverse hippocampal slices present a very well-preserved connectivity of the neuronal network (Lein, Barnhart, & Pessah, 2011) and have the advantage of allowing pharmacological agents to be quickly washed off (Skrede & Westgaard, 1971).

Here, we hypothesized that Cdc42 is crucial for persistent synaptic plasticity and underlies the setting of the synaptic tag. To test this hypothesis, we used a specific Cdc42 inhibitor, ML141, while recording extracellularly in acute transverse hippocampal slices. In particular, the specific aims of this work were:

- 1) Determine if Cdc42 is necessary for the induction of synaptic plasticity;
- 2) Determine if Cdc42 is necessary for the maintenance of synaptic plasticity;
- 3) Assess whether synaptic cooperation is possible in the absence of Cdc42 activity.

3. Methods

3.1. Animals

All experiments were performed using transverse hippocampal slices from weaned male Wistar Han rats (P21-P35), bred at the housing facility of the host institution (CEDOC/NOVA Medical School – Lisbon, Portugal). The procedures were approved by the Portuguese National Authority for Animal Health (DGAV) and are in accordance with the Decree-Law No. 113/2013 of 7 August (based on the EU Directive No. 2010/63 on the protection of animals used for scientific or educational purposes).

3.2. Slice preparation

The animals were decapitated under general anesthesia using isoflurane, after which the brain was quickly removed and immersed in ice-cold *cutting* artificial cerebrospinal fluid (ACSF), saturated with 95% O₂/5% CO₂ (**Table 1**).

Table 1 – Composition of ACSF used for the dissection procedure (“cutting ACSF”).

Components	Concentrations (mM)
NaCl	126
KCl	2.5
NaH ₂ PO	1.25
NaHCO ₃	26
MgCl ₂	5
CaCl ₂	1
Glucose	25

The hippocampi were then rapidly dissected and placed in a tissue slicer (Siskiyou MX-TS, see Figure 1 for a schematic representation) to obtain 400 µm-thick transverse hippocampal slices. Slices were allowed to rest in *cutting* ACSF at 32 °C for at least 1h before being transferred to a submersion chamber with *recording* ACSF (**Table 2**) for electrophysiological recordings.

Table 2 – Composition of ACSF used for the electrophysiological recordings (“recording ACSF”).

Components	Concentrations (mM)
NaCl	126
KCl	2.5
NaH ₂ PO	1.25
NaHCO ₃	26
MgCl ₂	2
CaCl ₂	2.8
Glucose	25



Figure 1 - Illustration of the tissue slicer used to obtain hippocampal slices. After dissection, the hippocampus is placed on an agarose bed (0.5% agarose, 0.9% NaCl), as depicted in the figure. Upon release of the trigger, a frame holding parallelly disposed tungsten wires is promptly lowered, separating the hippocampus in several transverse slices with 400 μm thickness and minimal tissue damage. Tungsten wire used had 0.02 mm in diameter, with 99.95% purity and no coating (Goodfellow Cambridge, Ltd.)

3.3. Electrophysiological recordings

Electrophysiological recordings were performed on acute hippocampal slices placed in a submersion-type recording chamber coupled with a stereo microscope, being continuously perfused by *recording* ACSF at 32 °C, saturated with 95% O₂/5% CO₂ throughout the entire duration of the experiments, circulating at a speed of approximately 2 mL/min.

Field excitatory postsynaptic potentials (fEPSPs) were recorded extracellularly in the *stratum radiatum* of the CA1 region (see **Figure 2**) using glass microelectrodes filled with 3 M NaCl immobilized with 0.5% agarose (tip resistance of 3-10 M Ω). Stimulating electrodes (monopolar epoxy-insulated tungsten electrodes; Science Products, GmbH, Germany) were placed on Schaffer collaterals projecting from CA3 to CA1 (S1 and S2, see **Figure 2**), positioned at an adequate distance from each other, allowing the stimulation of two independent sets of Schaffer collaterals. For experiments that required the activation of three pathways (e.g. synaptic capture experiments), a third stimulating

electrode was used to stimulate a different independent input in the antidromic direction (S3) that served as the control pathway. Schaffer collaterals were stimulated with 0.2-ms pulses.

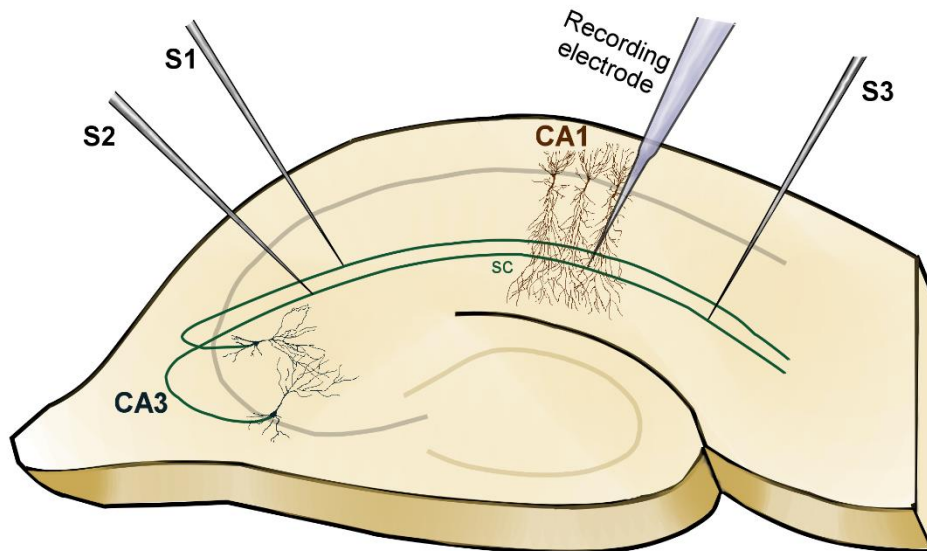


Figure 2 – Schematic representation of the position of recording and stimulating electrodes in the hippocampal slice. Stimulating electrodes S1 and S2 allow the activation of two independent sets of Schaffer collaterals. For experiments with three pathways, a third stimulating electrode (S3) was also used. The recording electrode was placed on the *stratum radiatum* of the CA1 region.

A paired-pulse facilitation (PPF) protocol was applied to assess pathway independence. Paired-pulse facilitation occurs when the same input is stimulated twice in rapid succession leading to a facilitation to the second of the two evoked responses (Creager, Dunwiddie, & Lynch, 1980). Thus, we can verify pathway independence by consecutively stimulating two different pathways (with a 30 ms inter-pulse interval). The test pulse frequency was 0.033 Hz and stimuli intensities were set to evoke 50% of maximal fEPSP slope. After a stable 20-min baseline recording, LTP was induced and fEPSPs were continuously recorded for 210 minutes.

3.4. Synaptic plasticity induction

A subset of performed experiments required the recording of two pathways. After the recording of a stable 20-min baseline, one of the pathways was randomly selected to receive LTP-inducing stimulation. The other pathway would serve as a control. The stimulated pathway could either receive a weak tetanic stimulation (**Figure 3**), capable only of inducing transient forms of plasticity, or a strong tetanic stimulation (**Figures 4, 5**

and 6), capable of inducing maintained forms of plasticity. The weak tetanic stimulation consisted of 2 trains of 25 pulses (100 Hz), with a 3-s interval between trains. The strong tetanic stimulation consisted of 5 trains of 25 pulses (100 Hz) with a 3-s interval between trains.

For both the weak and strong LTP experiments, we varied the timing of drug application with respect to LTP induction. Initially, we applied ML141 (a Cdc42 inhibitor) at a concentration of 5 μM for a 30-min time window, starting 10 minutes before and ending 20 minutes after LTP induction. We did this for both transient (**Figure 3**) and maintained (**Figure 4**) forms of LTP. For control experiments, only DMSO (0.005%) was applied.

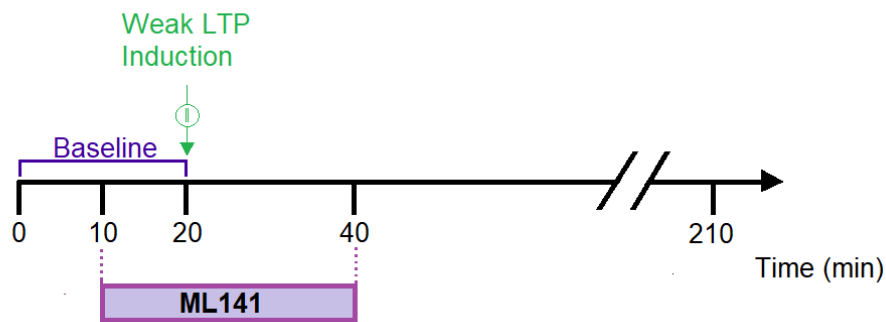


Figure 3 – Timing diagram for weak LTP experiments with drug application from 10 to 40 minutes. After a 20-min baseline, one of two pathways received weak tetanic stimulation. Application of ML141 (5 μM) starts 10 minutes before and ends 20 minutes after LTP induction. For control experiments, only DMSO (0.005%) was applied.

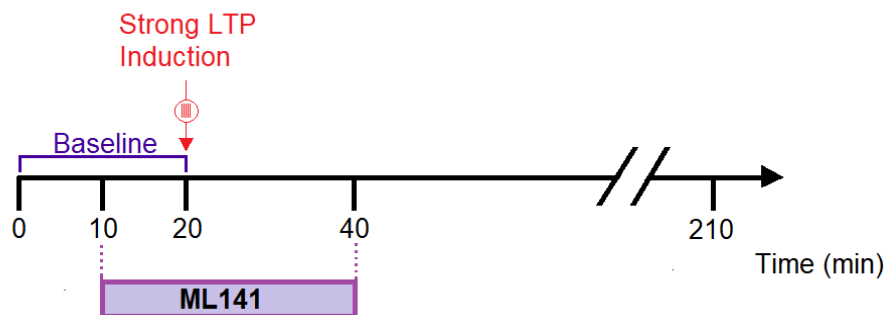


Figure 4 - Timing diagram for strong LTP experiments with drug application from 10 to 40 minutes. After a 20-min baseline, one of two pathways received strong tetanic stimulation. Application of ML141 (5 μM) starts 10 minutes before and ends 20 minutes after LTP induction. For control experiments, only DMSO (0.005%) was applied.

We varied the timing of drug application for maintained forms of LTP and performed experiments with a 30-min time window of drug application, starting either 40 minutes (60 to 90 minutes [Figure 5]) or 70 minutes (90 to 120 minutes [Figure 6]) after LTP induction with strong tetanic stimulation.

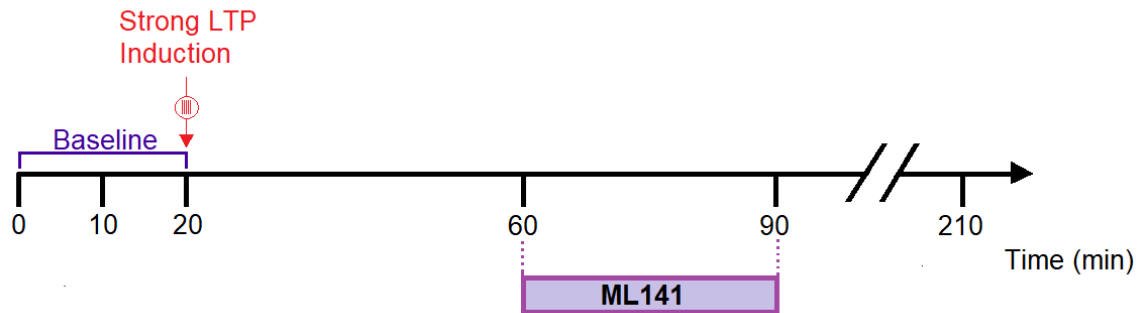


Figure 5 - Timing diagram for strong LTP experiments with drug application from 60 to 90 minutes. After a 20-min baseline, one of two pathways received strong tetanic stimulation. Application of ML141 (5 μ M) starts 40 minutes after LTP induction. For control experiments, only DMSO (0.005%) was applied.

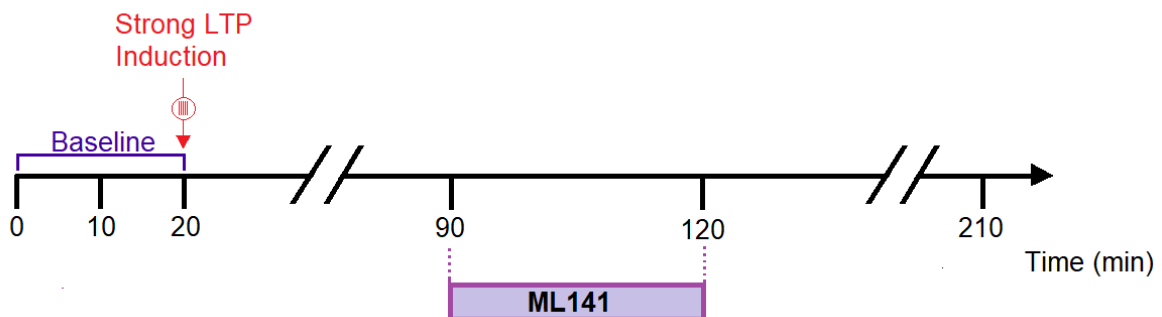


Figure 6 – Timing diagram for strong LTP experiments with drug application from 90 to 120 minutes. After a 20-min baseline, one of two pathways received strong tetanic stimulation. Application of ML141 (5 μ M) starts 70 minutes after LTP induction. For control experiments, only DMSO (0.005%) was applied.

To assess the effect of ML141 in the context of synaptic capture the recording of three pathways was required. After a 20-min baseline, one of the pathways received a weak tetanic stimulation and 30 minutes after the first LTP induction, a second pathway received a strong tetanic stimulation (Figure 7). A third pathway was recorded to serve as the control. Application of ML141 (5 μ M) started as soon as the first pathway received weak tetanic stimulation and ended when the second pathway received strong tetanic stimulation (20 to 50 minutes). For control experiments, only DMSO (0.005%) was

applied. We also recorded the behavior of a pathway that, under the same circumstances, received a weak tetanic stimulation in the absence of LTP-inducing tetanic stimulation in a second pathway (**Figure 8**).

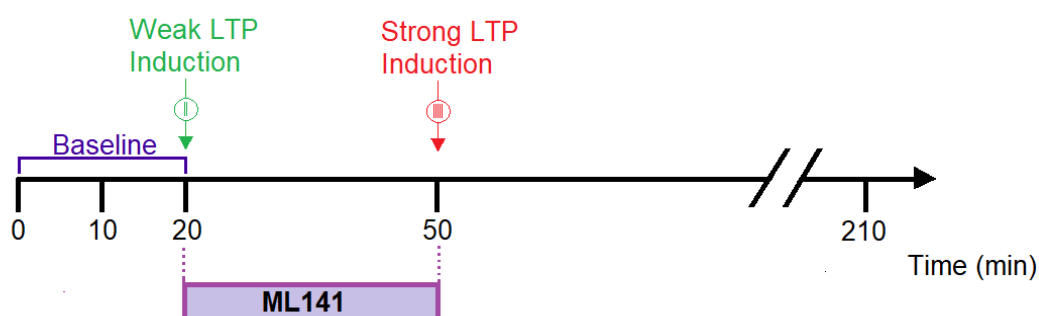


Figure 7 – Timing diagram for synaptic capture experiments. After a 20-min baseline, one of three pathways received a weak tetanic stimulation, inducing a transient form of LTP. At 50 minutes, 30 minutes after the induction of LTP in the first pathway, a second pathway received strong tetanic stimulation. Application of ML141 (5 μ M) between tetanic stimulations (20 to 50 minutes). For control experiments, only DMSO (0.005%) was applied.

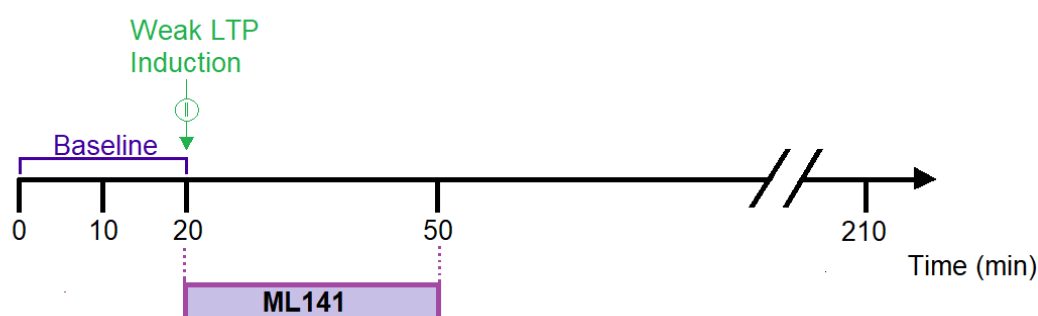


Figure 8 – Timing diagram for weak alone experiments. After a 20-min baseline, one of three pathways received a weak tetanic stimulation, inducing a transient form of LTP. The other two remaining inputs were also recorded and served as control pathways. Application of ML141 (5 μ M) started immediately after the induction of LTP and ended 30 minutes after. For control experiments, only DMSO was applied.

3.5. Drug treatment

ML141 (TargetMol, Boston, MA, USA) was dissolved in DMSO and diluted down to achieve the final concentration of 5 μ M (in 0.005% DMSO) and added to the circulating ACSF for the time periods mentioned above. For control experiments, only DMSO (0.005%) was added to the ACSF.

ML141 is a potent non-cytotoxic, specific and reversible non-competitive inhibitor of Cdc42 GTPase that was first identified in 2010 (Surviladze et al., 2010). This small

molecule is an allosteric inhibitor and its mechanism of action consists of selectively inhibiting nucleotide binding to the Cdc42 GTPase. Previously, it was demonstrated to be effective at very low micromolar concentrations (Hong et al., 2013).

3.6. Data acquisition and analysis

Electrophysiological data were collected using a Dagan IX2-700 amplifier (Dagan, Minnesota, USA) and band-passed filtered (low-pass filter: 1kHz, high-pass filter: 1Hz) using LHBF 48X from NPI Electronic, GmbH, Germany. Data were sampled using a Lab-PCI-6014 (National Instruments, Austin, TX, USA) with a sampling rate of 10 kHz and stored on a computer. Offline data analysis was performed using a customized executable program running on LabView language (LabView 8.2.1, National Instruments, Austin, TX, USA).

As a measure of synaptic strength, the initial slope of the evoked fEPSPs was calculated and expressed as percent changes from the baseline mean. Data were plotted using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

For the statistical analysis, the percentage of LTP decay was calculated by

$$\% LTP\ decay = \frac{(T_{initial} - T_{final})}{T_{initial}} \times 100$$

where $T_{initial}$ corresponds to the averaged values of fEPSP slope (as percent changes from the baseline mean) for the first 10-min after LTP induction or the 10-min period that immediately precedes drug application, according to experimental conditions ($T_1 = 20$ to 30 minutes; $T_2 = 50$ to 60 minutes; $T_3 = 80$ to 90 minutes), and T_{final} corresponds to the averaged values of fEPSP slope (as percent changes from the baseline mean) between 190 to 200 minutes (T_4). Experiments were rejected if the fEPSP slope values of the control pathway decayed below 80% of baseline values.

To test for group differences between percentage of LTP decay values across tested conditions we started by confirming normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene's test) of our data sets. For most cases we were only interested in comparing two groups and performed an unpaired two-tailed t-test. Whenever we were unable to assume normal distribution or homoscedasticity for one of the two groups, a non-parametric Mann-Whitney U test for independent samples was performed instead. For cases where we were interested in comparing more than two groups, we were also unable to assume normal distribution and performed a non-parametric Kruskal-Wallis test for independent samples with Dunn-Bonferroni multiple

comparison *post-hoc* test. All hypothesis tests mentioned above were performed with a 0.05 significance level using IBM SPSS Statistics (IBM, North Castle, NY, USA).

4. Results

4.1. Cdc42 inhibition blocks the induction of maintained forms of LTP

Previous studies from our laboratory have shown that interfering with actin cytoskeleton dynamics blocks the induction of persistent forms of synaptic plasticity (Fonseca, 2012; Szabó et al., 2016). Here, we assess the role of Cdc42, a member of the actin-regulating Rho GTPase family (Hall & Nobes, 2000), in LTP induction. To do this, we bath-applied ML141 (a selective Cdc42 inhibitor) at the time of LTP induction. For control experiments, only the drug vehicle was applied (DMSO).

While recording two independent pathways, we stimulated one of them with a weak high-frequency stimulation after a 20-min baseline and recorded the other as a control pathway to assess slice viability. The stimulated pathway displayed a transient form of LTP that returned to baseline values at the end of the 210-min recording (**Figure 9**). The induction of this transient form of LTP was not affected by application of ML141 (5 μ M), added to the ACSF 10 minutes prior to LTP induction and washed out 30 minutes later. For this set of experiments, an unpaired t-test (two-tailed; 95% confidence intervals) was conducted to compare the percentage of LTP decay in slices treated with ML141 and slices treated only with the drug vehicle. There was no significant difference in the percentage of LTP decay for ML141 (22.137 ± 6.537 %; $n = 7$) and DMSO (21.89 ± 2.996 %; $n = 9$) conditions; $t(14) = -0.036$; $p = 0.972$.

We repeated the same protocol using a strong high-frequency stimulation (see Methods). In this case, the stimulated pathway displayed a maintained form of LTP that was impacted by the application of ML141 (5 μ M) at the time of LTP induction (10 to 40 min; **Figure 10**). Control experiments were performed using only DMSO (0.005%) added to the ACSF instead of ML141. An unpaired t-test (two tailed; 95% confidence intervals) was conducted to compare the percentage of LTP decay in slices treated with ML141 and slices treated only with the drug vehicle. There was a significant difference in the percentage of LTP decay for ML141 (26.968 ± 5.707 %; $n = 8$) and DMSO (9.376 ± 3.840 %; $n = 10$) conditions; $t(16) = -2.643$; $p = 0.018$. Slice viability was never affected by drug application, as assessed by recording a second independent pathway. These results suggest that the induction of a maintained form of LTP is dependent on the activity of Cdc42.

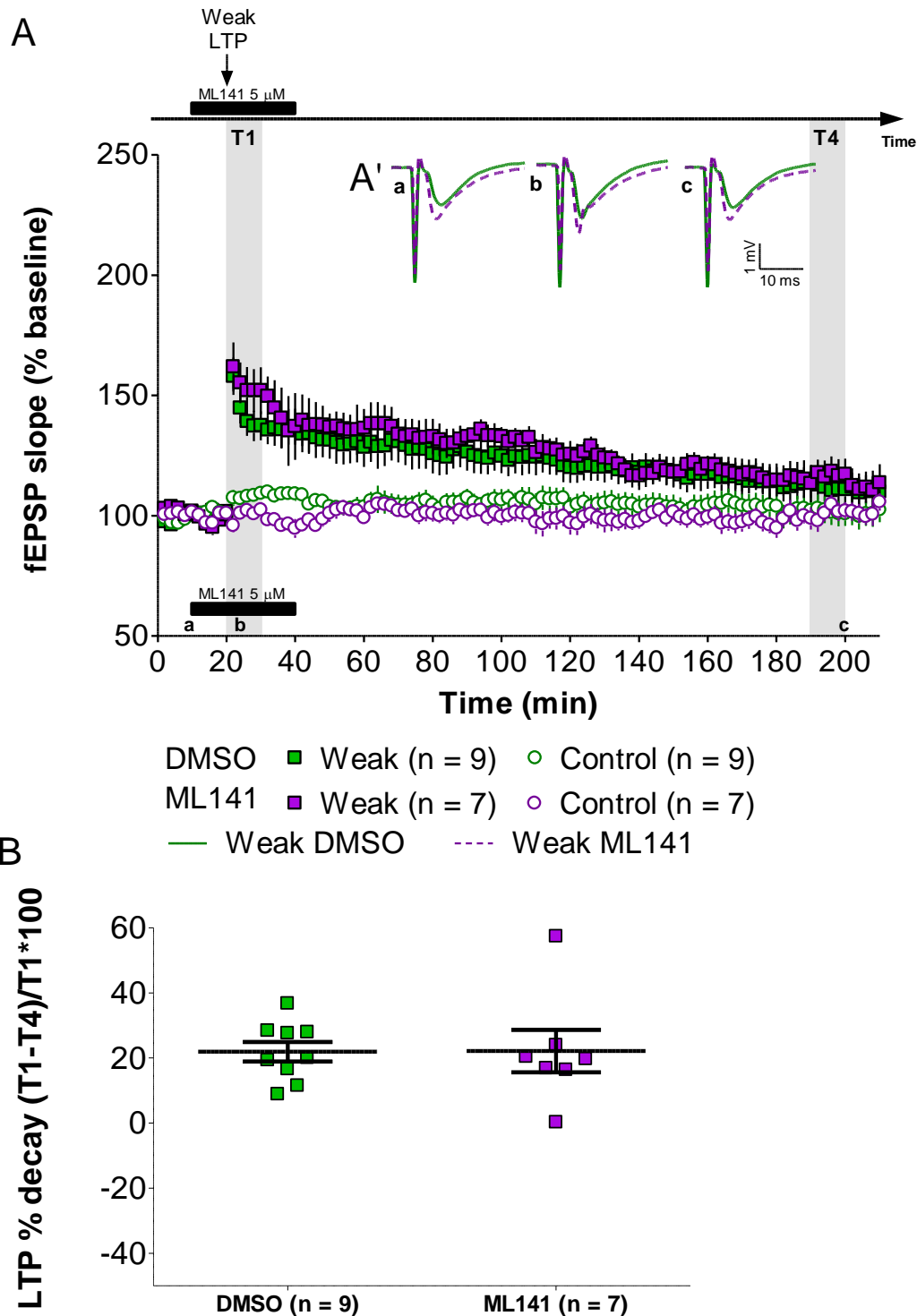


Figure 9 – Transient forms of LTP are not affected by Cdc42 inhibition. (A) Induction of LTP at 20 minutes with a weak high-frequency stimulation (HFS). ML141 (5 μ M ■) was bath-applied from 10 to 40 minutes. For control experiments, only DMSO (0.005%) was bath-applied (■). Control pathways were also recorded to assess slice viability (○). Data are represented as mean \pm SEM. (A') Representative average field excitatory postsynaptic potentials (fEPSPs) traces (average of three consecutive individual traces) for Weak LTP induced in the presence of ML141 (---) or DMSO only (—) at times indicated; scale: -1 mV, 10 ms. (B) Summary plot depicting the percentage of LTP decay for the time window analyzed (bold lines represent mean \pm SEM). No significant difference was found between conditions. T1 = 20 to 30 minutes; T4 = 190 to 200 minutes; “a”, “b” and “c” correspond to minutes 10, 20 and 200, respectively; n = number of slices.

4.2. The destabilization of LTP maintenance by Cdc42 inhibition is time-limited

We were interested in knowing if Cdc42 inhibition could disrupt the maintenance of persistent forms of LTP. To assess this, we performed two different sets of experiments: one where ML141 was applied 40 minutes after LTP induction (i.e. 60 to 90 minutes) and another where ML141 was applied 70 minutes after LTP induction (i.e. 90 to 120 minutes).

Two independent pathways were recorded and one of them was stimulated with strong high-frequency stimulation after a 20-min baseline. The other pathway was recorded as a control pathway to assess slice viability. ML141 (5 μ M) was bath-applied 40 minutes after LTP induction and washed-out 30 minutes later (**Figure 11**). The Cdc42 inhibitor destabilized the maintenance of persistent forms of LTP when applied during this period (60-90 minutes), when compared with the control experiments. Slice viability was not compromised, as assessed by the recording of the control pathway. For control experiments, only the drug vehicle (DMSO 0.005%) was added to the ACSF. For this set of experiments, we calculated the percentage of LTP decay between T2 (50-60 minutes) and T4 (190-200 minutes). To compare the percentage of LTP decay between conditions, we performed a non-parametric Mann-Whitney U test for independent samples (two-sided; 0.05 significance level), given that we were unable to assume homoscedasticity between both groups. There was a significant difference in the percentage of LTP decay for ML141 (23.314 ± 5.519 %; $n = 9$) and DMSO ($3,003 \pm 7.858$ %; $n = 10$) conditions; Mann-Whitney U = 83; $p = 0.002$. For experiments with drug application starting at 60 minutes, we considered the percentage of LTP decay between T2 (50-60 minutes) and T4 (190-200 minutes).

We next tested whether Cdc42 inhibition would affect LTP at even later periods of time, namely 70 minutes after LTP induction and washed-out 30 minutes later. Results show that the application of ML141 (5 μ M) did not interfere with LTP as compared to control experiments (**Figure 12**). For control experiments, only DMSO (0.005%) was added to the ACSF during that period (90-120 minutes). For this subset of experiments, we calculated the percentage of LTP decay between T3 (80-90 minutes) and T4 (190-200 minutes). An unpaired t-test (two-tailed; 0.05 level of confidence) was conducted to compare the percentage of LTP decay between the treatment and control conditions. There was no significant difference in the percentage of LTP decay for ML141 ($9.062 \pm$

2.555 %, n = 8) and DMSO (6.895 ± 2.438 %, n = 8) conditions; $t(14) = 1,311$; $p = 0.211$. These results suggest that maintained forms of LTP depend on Cdc42 activity for a limited period of time that extends beyond LTP induction.

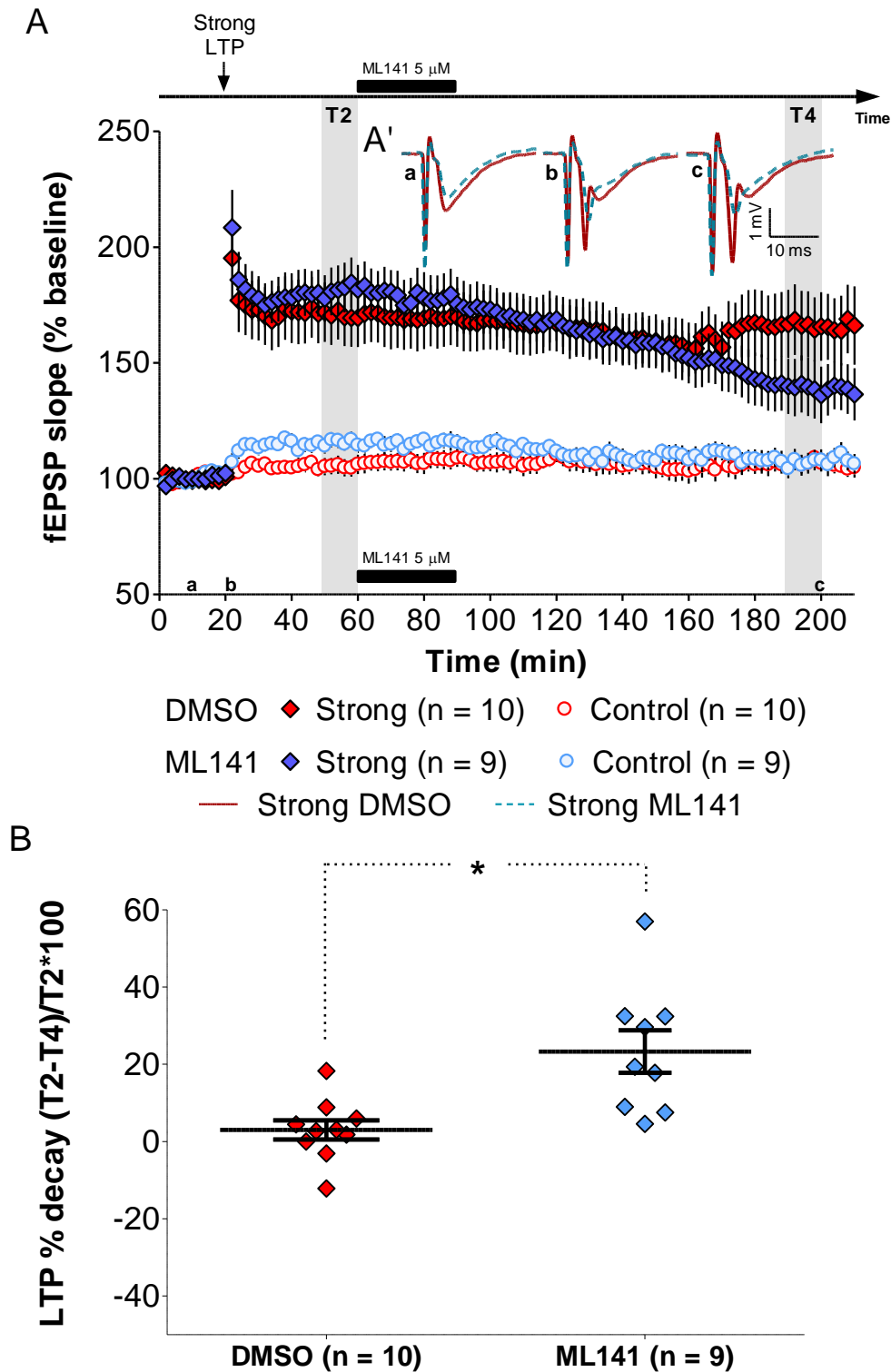


Figure 11 – Inhibition of Cdc42 destabilizes LTP maintenance (A) Induction of LTP at 20 minutes with a strong high-frequency stimulation (HFS). ML141 (5 μ M - \blacklozenge) was bath-applied from 60 to 90 minutes. For control experiments, only DMSO (0.005%) was bath-applied (\blacklozenge). Control pathways were also recorded to assess slice viability (\circ , \circ). Data are represented as mean \pm SEM. (**A'**) Representative average field excitatory postsynaptic potentials (fEPSPs) traces (average of three consecutive individual traces) for Strong LTP that received either ML141 (-----) or DMSO (—) treatment at times indicated; scale: -1 mV, 10 ms. (**B**) Summary plot depicting the percentage of LTP decay for the time window analyzed (bold lines represent mean \pm SEM). The application of ML141 impacted LTP maintenance as compared to the control group (* p = 0.002 by the Mann-Whitney U test; U = 83). T2 = 50 to 60 minutes; T4 = 190 to 200 minutes; “a”, “b” and “c” correspond to minutes 10, 20 and 200, respectively; n = number of slices.

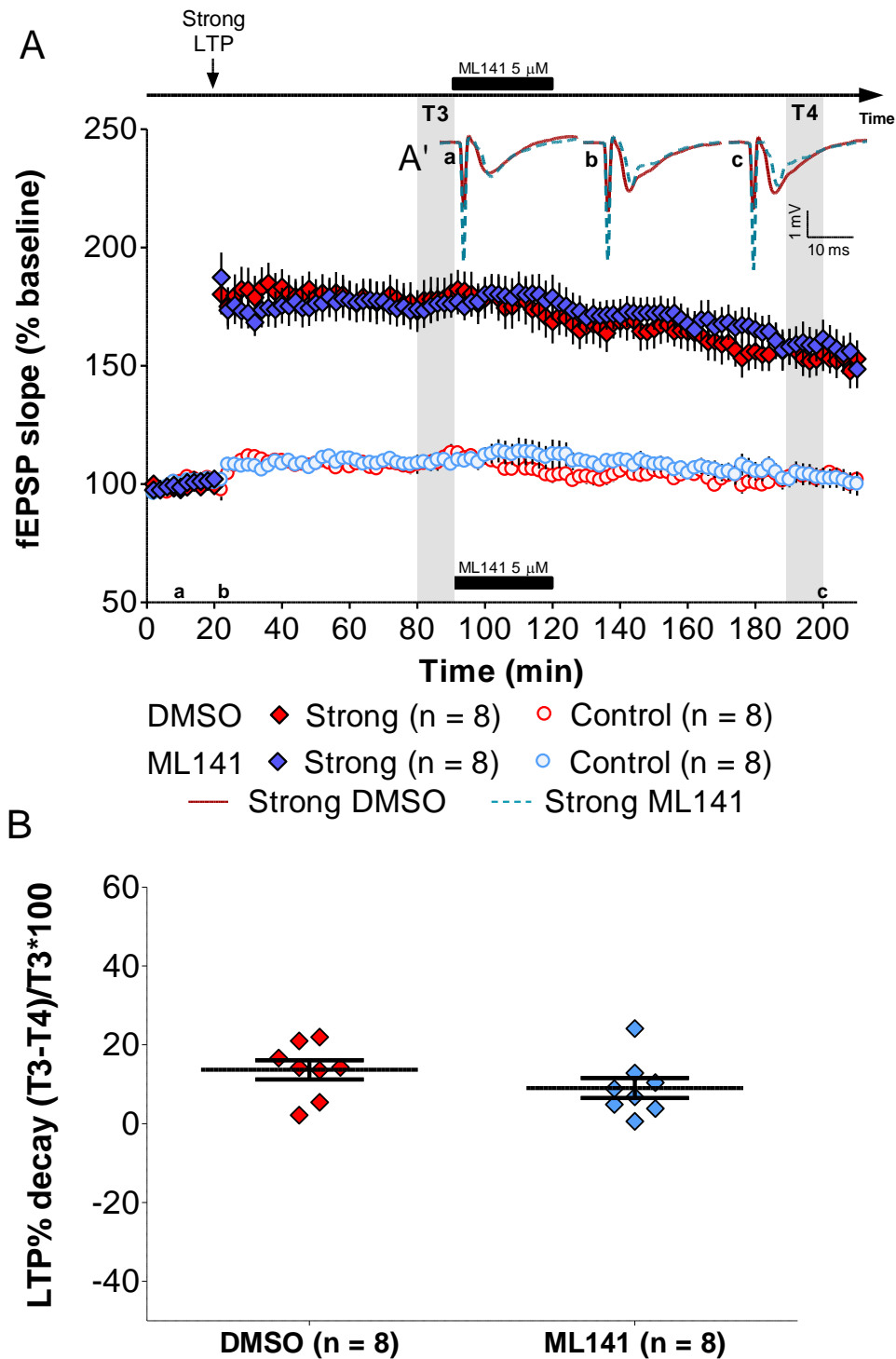


Figure 12 – LTP maintenance destabilization by Cdc42 inhibition is time-limited (A) Induction of LTP at 20 minutes with a strong high-frequency stimulation (HFS). ML141 (5 μ M \blacklozenge) was bath-applied from 90 to 120 minutes. For control experiments, only DMSO (0.005%) was bath-applied (\blacklozenge). Control pathways were also recorded to assess slice viability (\circ , \circ). Data are represented as mean \pm SEM. **(A')** Representative average field excitatory postsynaptic potentials (fEPSPs) traces (average of three consecutive individual traces) for Strong LTP that received either ML141 (dotted) or DMSO (solid) treatment at times indicated; scale: -1 mV, 10 ms. **(B)** Summary plot depicting the percentage of LTP decay for the time window analyzed (bold lines represent mean \pm SEM). The application of ML141 had no effect on LTP maintenance as compared to the control group ($t(14) = 1,311$; $p = 0.211$). T3 = 80 to 90 minutes; T4 = 190 to 200 minutes; “a”, “b” and “c” correspond to minutes 10, 20 and 200, respectively; n = number of slices.

4.3. Cdc42 inhibition interferes with synaptic capture

The results have shown that maintained forms of LTP were blocked when Cdc42 inhibition was performed for a short period of time after induction. It is then plausible to hypothesize that potentiated synapses might require the presence of Cdc42 for early plasticity mechanisms. One such mechanism could be the capture of plasticity-related proteins (PRPs) by potentiated synapses (Roger L. Redondo & Morris, 2011). For this subset of experiments, we required the recording of three independent pathways. After a 20-min baseline, one pathway (S1) was stimulated with a weak stimulation protocol. A second, independent pathway (S2) received a strong stimulation protocol 30 minutes later. A third input was also recorded to serve as a control pathway and assess slice viability (S3). Under control conditions (**Figure 13**), the pathway that received a weak stimulation (that would normally lead to a transient form of LTP) is able to convert its LTP into a stable, more persistent form of LTP due to the strong stimulation at S2.

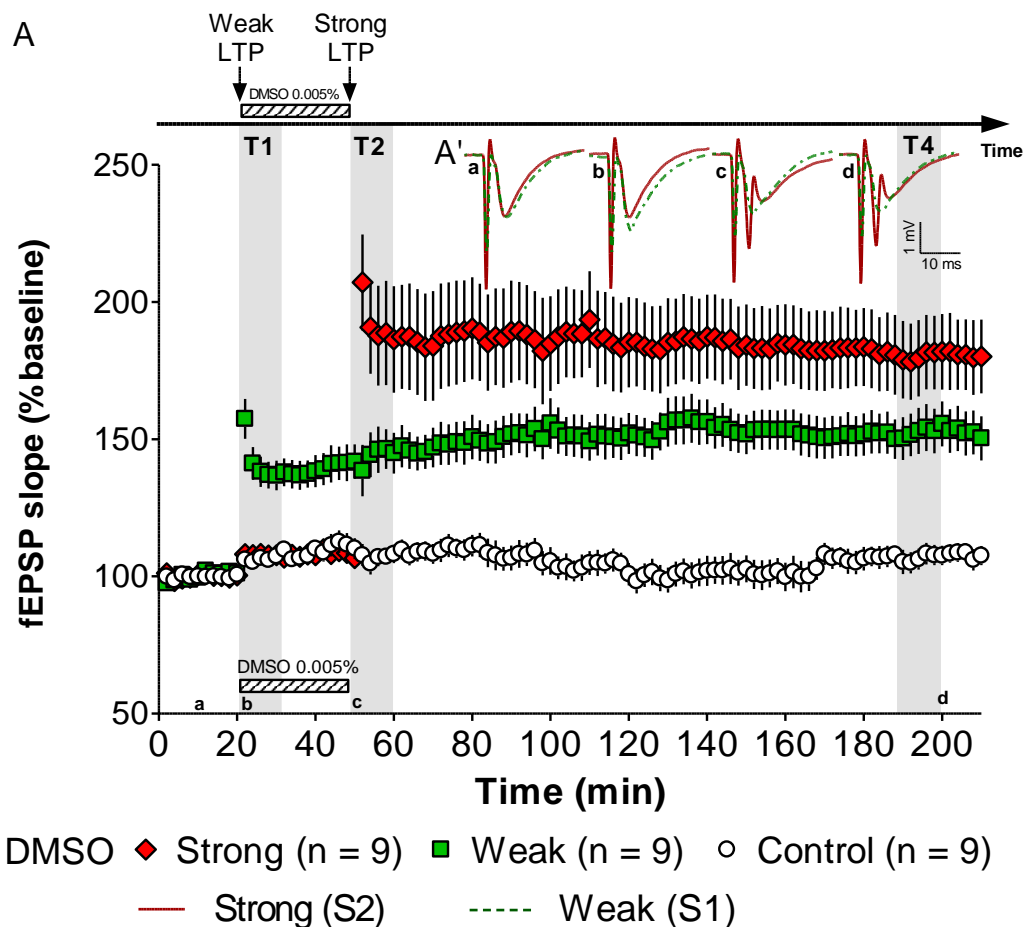


Figure 13 - Synaptic cooperation. (A) One pathway was stimulated with a weak tetanization protocol at 20 minutes (S1, ■). A second, independent pathway received a strong tetanization protocol 30 minutes later (S2, ♦). A control pathway was also recorded to assess slice viability (S3, ○). DMSO (0.005%) was added to the ACSF between both stimuli (from 20 to 50 minutes).

Data are represented as mean \pm SEM. (A') Representative average field excitatory postsynaptic potentials (fEPSPs) traces (average of three consecutive individual traces) for the pathway that received either weak (S1, ---) or strong (S2, —) tetanization at times indicated; scale: -1 mV, 10 ms. T1 = 20 to 30 minutes; T2 = 50 to 60 minutes; T4 = 190 to 200 minutes; "a", "b", "c" and "d" correspond to minutes 10, 20, 50 and 200, respectively; n = number of slices.

To assess the role of Cdc42 in synaptic capture, we added ML141 (5 μ M) to the ACSF between both stimulations, starting at 20 minutes and being washed-out at 50 minutes (Figure 14). In a separate group of experiments, we also recorded the behavior of a pathway that, under the same circumstances, received a weak tetanic stimulation at 20 minutes (S1, "weak alone"; Figure 14) in the absence of LTP-inducing tetanic stimulation in a second pathway.

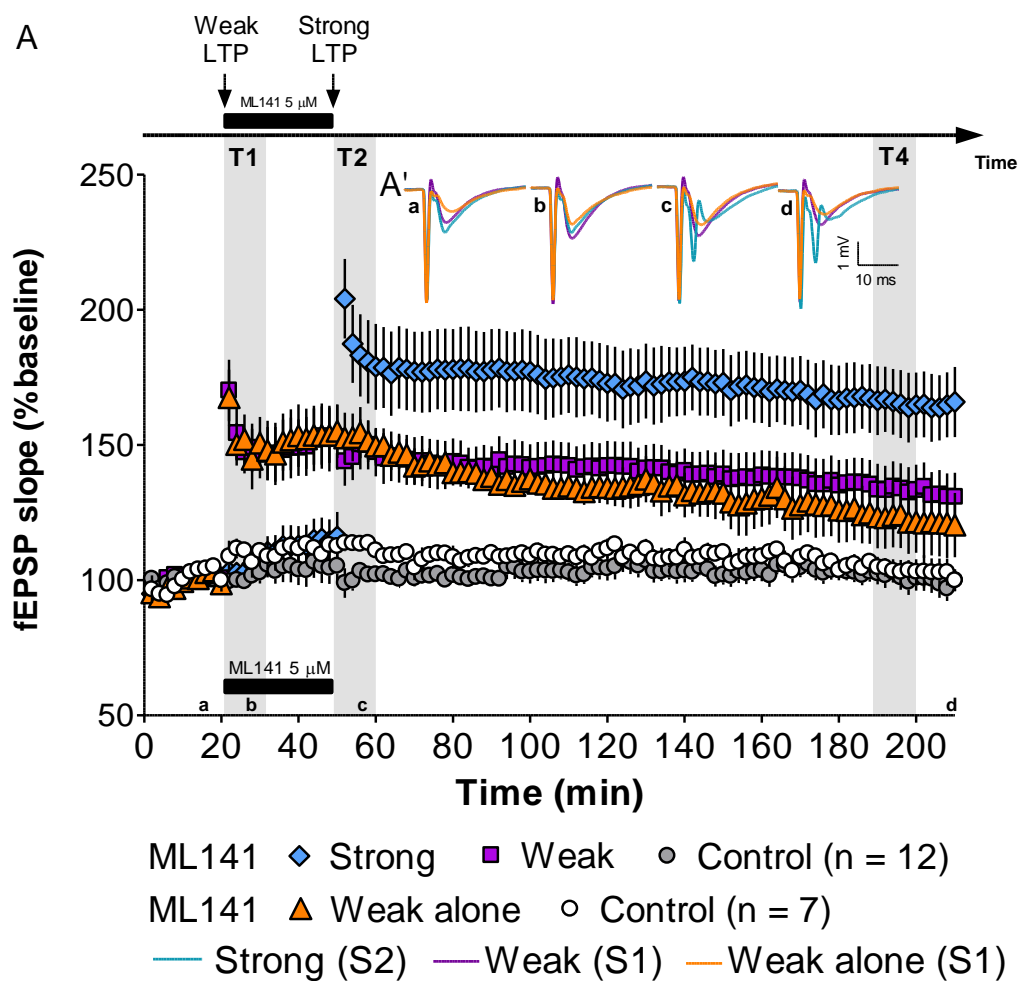


Figure 14 – Cdc42 inhibition interferes with synaptic capture. (A) One pathway was stimulated with a weak tetanization protocol at 20 minutes (S1, \blacksquare). A second, independent pathway received a strong tetanization protocol 30 minutes later (S2, \blacklozenge). A control pathway was also recorded to assess slice viability (S3, \bullet). In a separate group of experiments, only the first pathway was stimulated with a weak stimulus (S1, \blacktriangle) while control pathways were also recorded (\circ). ML141 (5 μ M) was added to the ACSF as soon as the first stimulus was applied (at 20 minutes) and washed-out 30 minutes later (at 50 minutes). Data are represented as mean \pm SEM. (A') Representative average field excitatory postsynaptic potentials (fEPSPs) traces (average of three consecutive individual traces) for the pathways that received either received weak (S1, —) or strong (S2, —) tetanization in the context of the cooperation experiments, or the pathways that received weak tetanization (S1, —) in the context of "weak alone" experiments at times indicated; scale: -1 mV, 10 ms. T1 = 20 to 30 minutes; T2 = 50 to 60 minutes; T4 = 190 to 200 minutes; "a", "b", "c" and "d" correspond to minutes 10, 20, 50 and 200, respectively; n = number of slices. 39

Regarding synaptic cooperation experiments, there is maintenance of LTP in both pathways in control conditions (**Figure 13**, DMSO 0.005%), as expected. However, if ML141 (5 μ M) is added to the ACSF between stimulations (20 to 50 minutes), the LTP expressed in the weakly stimulated pathway remains transient in nature and decays over time, similarly to the transient LTP expressed in the “weak alone” condition. This contrasts with what was observed in control conditions, where the LTP expressed in the weakly stimulated set of synapses persisted until the end of the recording (**Figure 15A**). To compare the percentage of LTP decay between conditions, a Kruskal-Wallis test for independent samples (two-way; 0.05 significance level) was conducted $H(2) = 11.559$; $p = 0.003$; $p < 0.05$, given that we were unable to assume normal distributions for all data sets. In order to make pairwise comparisons, a Dunn-Bonferroni multiple comparison *post-hoc* test was conducted. When compared to the control group (DMSO; $-8,328 \pm 4.829$ %; $n = 9$), both the application of ML141 ($12,533 \pm 3.953$ %; $n = 12$) and the “weak alone” group (17.4274 ± 10.499 %; $n = 7$) show significantly higher percentage of LTP decay ($p = 0.018$ and $p = 0.006$, respectively). There was no significant difference in percentage of LTP decay between the “weak alone” group and the ML141 group ($p = 1.000$). These results show that inhibiting Cdc42 lead to an inefficient capture of synaptic plasticity at the weakly stimulated pathway, suggesting that Cdc42 activity is required for synaptic capture.

Treatment with the Cdc42 inhibitor had no effect on the pathway that received a strong tetanization at 50 minutes (immediately after ML141 treatment). The pathway expressed an equally persistent form of LTP in ML141-treated slices as compared to the control group (**Figure 15B**). To compare the percentage of LTP decay between conditions, we performed a non-parametric Mann-Whitney U test for independent samples (two-tailed; 0.05 significance level) given that we were unable to assume that both data sets followed a normal distribution. There was no significant difference in the percentage of LTP decay for ML141 (10.408 ± 2.660 %; $n = 12$) and DMSO ($3,716 \pm 4.555$ %; $n = 9$) conditions; Mann-Whitney $U = 70$; $p = 0.256$.

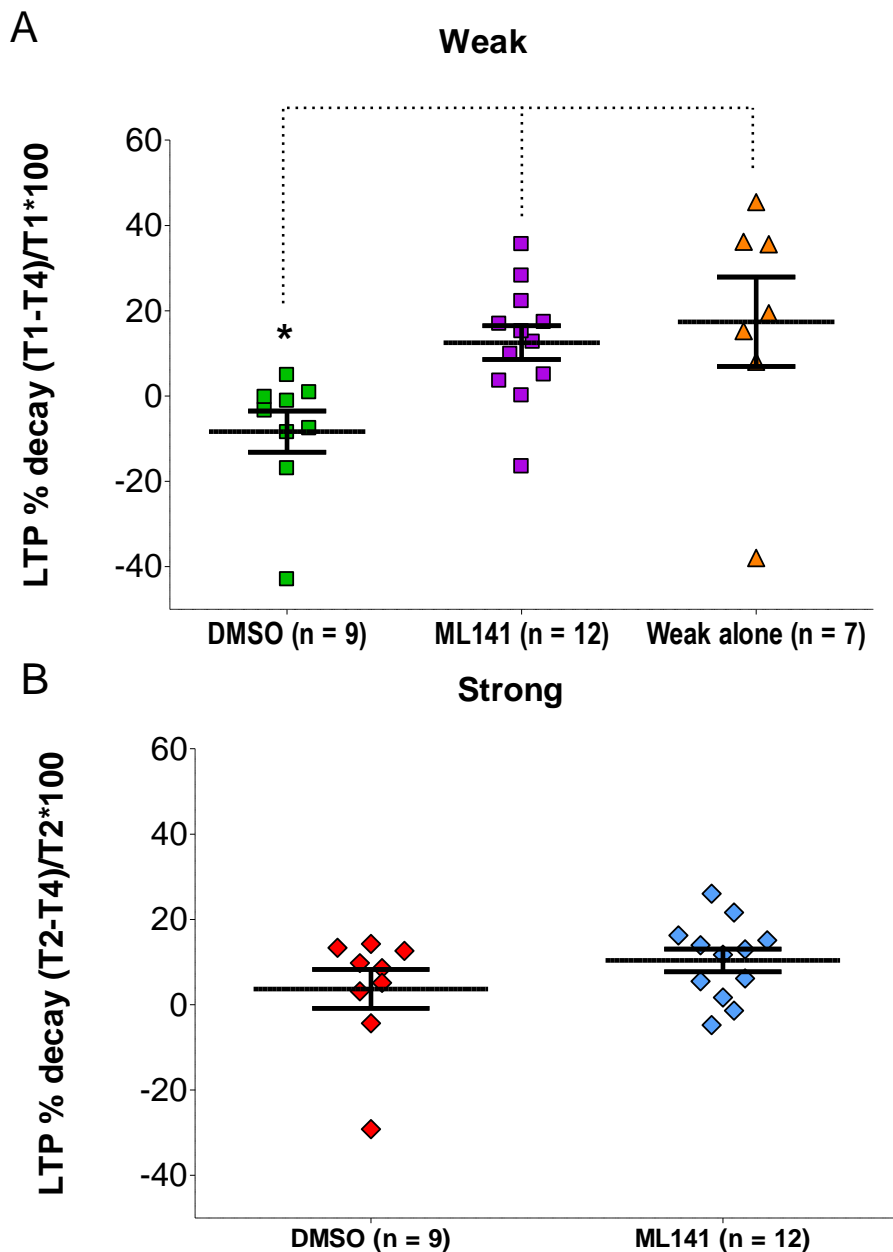


Figure 15 – Percentage of LTP decay for synaptic capture experiments. (A) Summary plot depicting the percentage of LTP decay for the weakly stimulated pathway. The application of ML141 significantly increased the percentage of LTP decay when compared to the control group (Kruskal-Wallis test, $H(2) = 11.559$; $p = 0.003$; adjusted significance level for multiple comparisons: $p = 0.018$). The percentage of LTP decay was considered between T1 (20-30 minutes) and T4 (190-200 minutes). (B) Summary plot depicting the percentage of LTP decay for the strongly stimulated pathway. The application of ML141 had no effect on LTP as compared to the control group; Mann-Whitney $U = 70$; $p = 0.256$. The percentage of LTP decay was considered between T2 (50-60 minutes) and T4 (190-200 minutes). Bold lines represent mean \pm SEM; $n =$ number of slices.

5. Discussion

In the present work we were able to assess the role of a Cdc42, a well-known Rho GTPase and regulator of actin dynamics, in the induction and maintenance of long-lasting forms of synaptic plasticity. We used ML141, a non-competitive inhibitor, highly selective for Cdc42 (Surviladze et al., 2010) that acts by blocking the binding of Cdc42 GTPase to guanine nucleotides, such as guanosine triphosphate (Hong et al., 2013). Without the ability to be in the GTP-bound conformation, Cdc42 is inactive. Moreover, being what is called a “conventional” Rho GTPase, Cdc42 is the target of activation by a number of GEF regulatory proteins. However, in the presence of ML141, GEFs are also unable to activate Cdc42, since there is no nucleotide binding to mediate.

Several naturally occurring toxins that directly bind to actin have now been isolated from marine sponges and fungi and provide important tools in the study of how actin is involved in various cellular functions (Allingham, Klenchin, & Rayment, 2006). These toxins may bind to filamentous actin (F-actin) and exert different effects on actin dynamics. Cytochalasins, for instance, are fungal metabolites that bind to the barbed (plus) end of actin filaments, therefore preventing their polymerization (Cooper, 1987). Phalloidin, found in the death cap mushroom (*Amanita phalloides*), binds and stabilizes F-actin, preventing its depolymerization (Cooper, 1987). Jasplakinolide, isolated from a marine sponge, stabilizes actin filaments and shifts the F-actin/G-actin equilibrium towards the F-actin (Bubb, Senderowicz, Sausville, Duncan, & Korn, 1994). Toxins may also sequester G-actin, prevent polymerization and enhance depolymerization, as is the case of latrunculins (Morton, Ayscough, & McLaughlin, 2000). Earlier studies that coupled electrophysiology with the use of these substances have reported that the inhibition of actin polymerization suppresses LTP induction, maintenance and tagging in the rodent hippocampus (Chen et al., 2007; C.-H. Kim & Lisman, 1999; Krucker, Siggins, & Halpain, 2000; Ramachandran & Frey, 2009). Previous results from our laboratory group have shown a critical role of actin dynamics in the tagging and capture of PRPs in both LTP and LTD (Fonseca, 2012; Szabó et al., 2016).

Here, we show that the inhibition of Cdc42 at the time of induction of synaptic plasticity leads to the blockade of persistent forms of LTP. In addition, it was also shown that Cdc42 inhibition destabilizes LTP maintenance when ML141 was applied at later time periods. This destabilization occurred in a time-limited manner: Cdc42 inhibition only interfered with LTP maintenance when ML141 was applied up until 70 minutes after LTP induction. These results suggest a Cdc42-dependent early mechanism for induction and maintenance of LTP. Since Cdc42 activation promotes actin polymerization, we propose that Cdc42 inhibition may interfere with the maintenance of LTP by promoting actin depolymerization. It is interesting to note that transient forms of LTP were not

affected by Cdc42 inhibition. Moreover, our results from the synaptic tagging experiments suggest that Cdc42 is necessary for tagging and that inhibiting its activity lead to an impaired capture of macromolecules necessary for the maintenance of long-lasting forms of LTP at the synapse. It is likely, however, that Cdc42, RhoA and Rac1 are all necessary for functional and structural synaptic plasticity and that their activation complement each other in order to transduce extracellular signals that, through activation of NMDA receptors and BDNF and CaMKII signalling, lead to synaptic remodeling and scaffolding of new proteins necessary for the proper strengthening of connections between synapses (Duman & Tolias, 2016; Hedrick et al., 2016; Hedrick & Yasuda, 2017). In the future, it would be interesting to know, perhaps with the aid of fluorescence resonance energy transfer-based imaging, the subcellular localization and timing of interaction between these Rho GTPases and their downstream effectors after the induction of synaptic plasticity.

In a 2014 paper from Bosch and colleagues (Bosch et al., 2014), the structural and molecular remodeling of individual dendritic spines during synaptic plasticity was studied using two-photon glutamate uncaging. The authors divided sLTP into two temporal phases. The first, initial phase consisted of an actin cytoskeleton remodeling phase (<7 min), accompanied by a rapid increase of actin and cofilin concentration in the spine. Arp2/3 is also enriched in the spine in the first few minutes. Upon sLTP induction, cofilin showed a rapid increase in concentration within the first 20 s after stimulation and remained highly enriched in the spine for up to 30 minutes, suggesting that it plays a major role in F-actin remodeling. The authors postulate that cofilin and Arp2/3 (both downstream of Cdc42) might act synergistically to create new branched filaments that will participate in the maintenance of synaptic plasticity and the delivery of new proteins to the synaptic membrane. At the same time, the concentration of proteins that could stabilize and compete with cofilin and Arp2/3 for F-actin binding sites is transiently reduced (e.g. CaMKII β , drebrin) (K. Kim et al., 2015; K.-I. Okamoto, Narayanan, Lee, Murata, & Hayashi, 2007). Thus, there is a short time window in which the actin cytoskeleton becomes susceptible to remodeling. The second, stabilization phase (>7 min) was associated with the formation of a stable complex of cofilin with F-actin. Cofilin was persistently retained at potentiated spines. The bound cofilin population was still detectable 60 minutes after sLTP induction. Cofilin activity has been described as being dependent on concentration (Andrianantoandro & Pollard, 2006). At a low stoichiometric ratio of cofilin, single cofilins bind and sever actin filaments. Higher concentrations of cofilin bind cooperatively to F-actin, promoting the release of inorganic phosphate without severing the filaments. In fact, if the concentration of cofilin is high enough, its activity

might lead to actin filament assembly (Andrianantoandro & Pollard, 2006). Thus, cofilin has a bidirectional effect on actin dynamics - it can either aid in F-actin disassembly or F-actin assembly – and has shown to be a necessary protein for the consolidation of functional and structural LTP (Bosch et al., 2014; Rust et al., 2010). As such, cofilin must be inactivated in time, not only to be retained in the spine, but also to avoid reverting the polymerizing trend toward depolymerization during a phase of stabilization. Interestingly, PAK and cofilin were shown to be phosphorylated within a time window of 2-7 minutes after LTP induction (Chen et al., 2007). The Rho GTPase Cdc42 acts upstream of the well-characterized PAK-LIMK pathway, that regulates cofilin activity through its phosphorylation (Nakahata & Yasuda, 2018). The inactivation of cofilin is mainly carried out by phosphorylation of its serine 3 residue by LIM kinases (LIMK) and activated through dephosphorylation by Slingshot (SSH) phosphatases, although other regulators do exist (Van Troys et al., 2008). Upon the induction of synaptic plasticity, unphosphorylated cofilin is initially concentrated at dendritic spines but it needs to be phosphorylated for long-term retention (Bosch et al., 2014). Moreover, interfering with PAK-LIMK signalling was shown to lead to an impaired persistent retention of cofilin and a reduction in spine enlargement during sLTP (Bosch et al., 2014). Thus, Cdc42 is upstream of a signaling pathway that is heavily implicated in structural plasticity in spines.

Overall, our results support the hypothesis that, by regulating the actin cytoskeleton, Cdc42 interferes with synaptic plasticity. This and other studies on the mechanisms through which the actin cytoskeleton is remodeled as a consequence of neuronal activity may have profound implications in the way we understand learning and memory processes (I. H. Kim, Wang, Soderling, & Yasuda, 2014; Zhang, Li, Wang, Liu, & Zhong, 2016). Besides, these observations may provide important therapeutic targets for neuropsychiatric diseases for which there are already identified disfunctions regarding the actin cytoskeleton or its complex network of regulators (Bamburg & Bernstein, 2016; Deng et al., 2016; Ide & Lewis, 2010; Yan, Kim, Datta, Lewis, & Soderling, 2016).

6. References

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