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Understanding the molecular mechanisms underlying fear extinction and anxiety disorders: key role of protein-phosphatase 1

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Presented by

Osvaldo Mirante

M.Sc., University of Lausanne, Switzerland

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Citizen from Lausanne, Switzerland

Accepted on the recommendation of

Prof. Isabelle M. Mansuy, examiner

Prof. Urs Gerber, co – examiner

Prof. Ron Stoop, co – examiner

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Le Bonheur est une bulle de savon qui change de couleur comme l'iris et qui éclate quand on la touche.

Balzac

Si vide d’espoir est le monde du dehors que deux fois plus précieux m’est le monde de dedans.

Emily Brontë

Fear was born in the very same moment as mankind. And since we will never be able to master it, we will have to learn to live with it – just as we have learned to live with storms. Therefore, although fear is a part of life, never let it control you.

Paulo Coelho

A mes parents Patrizia et Domenico et à mes frères Claudio et Alessandro

… … …

Je t’aime toi

… … …

1 Abstract

The brain observes, detects, learns, and remembers signals coming from our external environment. In particular, it senses danger, and once detected, the related sensory signal, be it auditory, visual or somatosensory, triggers synaptic plasticity, enabling the brain to learn and remember the event. During Pavlovian fear conditioning, animals learn to associate a negative stimulus, such as a mild foot-shock, with a neutral stimulus, like a novel sound or context. This association leads to long-lasting changes in the animals' behavior upon re-exposure to the neutral stimuli that, in rodent, is expressed by a "freezing" fear posture. Fortunately, these fear memories, in rodent and human, extinguish with repeated exposure to the neutral stimuli in absence of the negative stimulus. If fear extinction were impaired, however, one would live every day in an anxious state of exaggerated fear. Humans with post-traumatic stress disorder suffer from a persistent fear memory that is resistant to extinction. Although therapies based on extinction already exist, their efficiency is low and the majority of patients experience relapse.

This thesis proposes that a detailed investigation of the molecular mechanisms underlying the synaptic plasticity of fear extinction will enable better treatment for anxiety disorders, and uses electrophysiology to step modestly in that direction.

Fear memory formation and extinction occur principally in the amygdala, a brain area localized within the temporal lobe. The amygdala receives sensory stimuli primarily from the thalamus and cortex and long-term potentiation of thalamo- and cortico-amygdalar synaptic transmission is believed to underlie

fear memory formation. In contrast, fear extinction is thought to involve long-term depression of these circuits. My doctorate focused on understanding the molecular pathways underlying long-term depression of thalamo- and cortico-amygdalar projections. I found that thalamic and cortical inputs carry distinct molecular pathways, such as distinct receptors localized at different sites of long-term depression induction.

2 Résumé

Le cerveau observe, détecte, apprend et se souvient de signaux provenant de notre environnement externe. En particulier, le danger. Une fois détecté, le signal sensoriel lié au danger, qu'il soit auditif, visuel ou somato-sensoriel, déclenche une plasticité synaptique, permettant au cerveau d'apprendre et de se souvenir de l'événement. Lors du conditionnement Pavlovien de la peur, les animaux apprennent à associer un stimulus négatif, tel qu'un léger choc électrique aux pattes, avec un stimulus neutre, comme un nouveau son ou contexte. Cette association conduit à des changements durables dans le comportement des animaux en cas de réexposition aux stimuli neutres qui, chez les rongeurs, est exprimée par une posture «blocage » liée à la peur.

Heureusement, ces mémoires liées à la peur, chez les rongeurs et les humains, s'estompent lorsque les expositions des stimuli neutres sont répétées en absence du stimulus négatif. Cependant, si l'extinction de la peur est altérée, on vivrait tous les jours dans un état anxieux de peur exagérée. Les humains atteints de stress post-traumatique souffrent d'une mémoire persistante de la peur et qui est résistante à l'extinction. Bien que les

thérapies basées sur l'extinction existent déjà, leur efficacité est faible et la majorité des patients traités ont présentés une rechute.

Cette thèse propose qu'une analyse détaillée des mécanismes moléculaires sous-jacents à la plasticité synaptique de l'extinction de la peur permettra un meilleur traitement pour les troubles de l'anxiété, et utilise l'électrophysiologie comme étape modeste allant dans cette direction.

La formation de la mémoire de la peur et de son extinction se produisent principalement dans l'amygdale, une zone du cerveau localisée au sein du lobe temporal. L'amygdale reçoit des stimuli sensoriels essentiellement du thalamus et du cortex, et la potentialisation à long terme de la transmission synaptique thalamo- et cortico-amygdalien est considérée être à la base de la formation de la mémoire de la peur. En revanche, on considère que l'extinction de la mémoire de la peur implique une dépression à long terme de ces circuits. Mon doctorat se focalise sur la compréhension des mécanismes moléculaires sous-jacents la dépression à long terme des projections thalamo- et cortico-amygdalien. J'ai trouvé que les voies thalamiques et corticales impliquent des voies moléculaires distinctes, tels que différents récepteurs localisés sur différents sites d'induction de dépression à long terme.

3 General Introduction

3.1 Fear and anxiety disorders

Fear is an emotional state related to behavioral and physiological responses induced by a specific stimulus, such as pain or the threat of danger. Fear is the most highly conserved emotion that occurs throughout the animal kingdom [1,2]. It is a basic survival mechanism that allows an organism to either fight or escape (flight) from a danger [1]. However, in extreme cases of fear, such as horror and terror, fear might be maladaptive, unproductive, and even harmful [3]. An example illustrating a severe outcome of extreme fear, such as the consequences of exposure to a traumatic event, is human anxiety disorders [4].

Anxiety disorders, also known as fear-related disorders, are some of the most prevalent psychiatric illnesses, affecting about 15% of the population worldwide [4]. They include panic disorder, social anxiety disorder, specific phobias, obsessive-compulsive disorder, generalized anxiety disorder and post-traumatic stress disorder (PTSD) [4-7].

3.1.1 Post-traumatic stress disorder: alteration of the process of fear

PTSD is by far the most studied [5] of the anxiety disorders, as it is the only major mental disorder for which there is a clear understanding of its cause[8]. For example, PTSD can develop after exposure to any psychologically traumatic event, from combat exposure, natural disasters, domestic violence (during childhood or adulthood), life-threatening accidents [8-10], and mobbing [11].

PTSD belongs to the family of anxiety disorders, because in most cases the stimulus that produced the initial fear response is no longer present as an active threat. A key symptom in patients with anxiety disorders is that they possess exaggerated fear reactions to situations that would in healthy humans be considered safe [3]. This is caused by an alteration of the process called fear extinction, meaning that the fear memory of the traumatic event is resistant to decay [3,5,8]. A perturbation in fear extinction is the cause of some of the key symptoms of PTSD, including intrusive memories of the traumatic event, such as re-experiencing the event during flashbacks and nightmares [3,8], avoidance of reminders of the traumatic event, hyper-arousal and emotional numbing [3,8]. These symptoms lead to sleep disorders, personality changes, cognitive impairments and destructive forms of self-medication [9,10]. It is therefore crucial to understand the mechanisms underlying learning and memory of fear, since this may lead to more efficient treatments for anxiety disorders, and for PTSD in particular.

3.2 Studying fear memory and extinction

Of the various models available to study fear learning and memory [12], Pavlovian (or classical) fear conditioning is commonly considered a gold-standard in the field, since it has proven both successful and useful for understanding the underlying mechanisms of normal and pathological fear responses [1,3,5,8]. Pavlov based his behavioral paradigm on the universal concept of the fear-reflex: an aversive/fearful stimulus gives rise to a fear response [13]. Since all species have conserved a fear-reflex as a primary

survival mechanism, Pavlovian fear conditioning can be applied on a multitude of different animals, from rodents to humans [8,14-16].

3.2.1 Associative fear memory in both rodents and humans

In rodents, classic fear conditioning consists of presenting the animal with an emotionally neutral conditioned stimulus (CS), such as a tone, associated with an aversive unconditioned stimulus (US), such as an electrical foot-shock. The foot-shock then elicits spontaneous unconditioned fear responses (UCRs), such as freezing or increased heart rate. After one or several CS-US pairings, the CS alone is able to elicit conditioned fear responses (CRs), suggesting that the rodent has learned that the CS is followed by the US, leading to the formation of an associative fear memory [1,8]. The commonly measured CR is related to defensive behaviors such as freezing. Freezing is a behavior observed in rodent species in their natural environment, and is thought to be a crucial fear response needed for survival in an environment where even small movements may attract predators' attention. For example, in humans, encountering a snake in a field will initially evoke a freezing response, followed by flight (running away) or vocalization (screaming)[17]. Other types of CRs often analyzed include autonomic nervous system (ANS) responses, such as increased blood pressure and heart rate, neuroendocrine responses (the release of hormones from pituitary and adrenal glands), alterations in pain sensitivity (analgesia) and reflexes (fear-potentiated startle and eye blink responses)[1,2,18].

3.2.2 Fear extinction in both rodents and humans

Normally the previously acquired conditioned fear memory progressively extinguishes over time after a persistent absence of the aversive signals (US). This phenomenon is known as the extinction of fear memory and can also be studied using Pavlovian fear conditioning. To illicit fear extinction, the CS (the tone) is repetitively applied in the absence of the US (electrical foot-shock). In this configuration, the CS alone will elicit a decrease in expression of the CRs, since the tested animal learns that the CS is no longer paired with the US, thereby leading to the extinction of the conditioned fear memory [1,14,19]. Pavlovian fear conditioning can also be applied to humans [8,14,20,21]. In humans, the CS is often based on the presentation of images or sounds related to the trauma the patient has been faced to in his past, the US is often mediated by an electric shock to the wrist or fingers, and the observed CRs are mainly the heart rate, startle effect or skin conductance [5,7,8,14,20,22].

3.2.3 PTSD: normal fear memory and fear extinction impairment

Using the parameters cited above, PTSD patients show an abnormal resistance to fear extinction with a normal fear memory compared to healthy patients [3,5-8,10,15,20,21,23]. Similar to humans with PTSD, rats that have been exposed to a traumatic event, such as a predator threat (placement of the rats on soiled cat litter for 10 min) before fear conditioning exhibit impaired fear extinction and a normal fear memory [24].

In summary, classical fear conditioning has been extensively investigated in rodents, and is increasingly applied on humans [20]. The findings from rodents' research have been supported and extended in humans, by using

neuropsychological and neuroimaging methodologies [22]. Research on fear conditioning helped to describe a brain circuitry thought to be responsible for the acquisition, expression and the extinction of fear memory. A goal of fear research is to understand how to treat the potentially destructive effects of anxiety disorders, such as PTSD, in humans. As an outcome, fear extinction has been identified as a key target for future potential treatment for anxiety disorders. Thus, understanding the brain anatomy involved in fear extinction is one crucial step towards a therapeutic application of PTSD research.

3.3 Understanding the brain anatomy involved in fear extinction

The identification of the principal brain structures involved in fear circuitry, as well as their functionality, has been possible due to the use of the fear-conditioning paradigm in combination with other techniques, such as electrophysiology, e.g. extracellular field and whole-cell patch clamp recordings, and neuroimaging studies, including functional magnetic resonance imaging (fMRI) and positron electron microscopy (PET). In rodents and humans, the three main brain areas involved in fear circuitry are the amygdala, the sensory thalamus and the sensory cortex.

3.3.1 The anatomy and circuitry of fear memory

In fear conditioning, the auditory and somatosensory information emitted from the CS and US, respectively, are first received by the sensory thalamus. From the thalamus, the sensory information is forwarded either directly to the amygdala through the internal capsule (ic, Figure 1) or indirectly, via projections to the sensory cortical areas (auditory cortex and somatosensory cortex for auditory and somatosensory information, respectively).

The sensory cortex sends afferents to the amygdala via the external capsule (ec). The lateral nucleus of the amygdala (LA, see The amygdala) integrates the CS and US related information and projects to the central nucleus of the amygdala (CE), which is responsible for eliciting fear responses. For doing so, the CE afferents project to other brain regions, such as the hypothalamus (HYP) and the periaqueductal gray (PAG), which control the blood pressure and induce freezing behavior, respectively [1,2,25-33] (Figure 1).

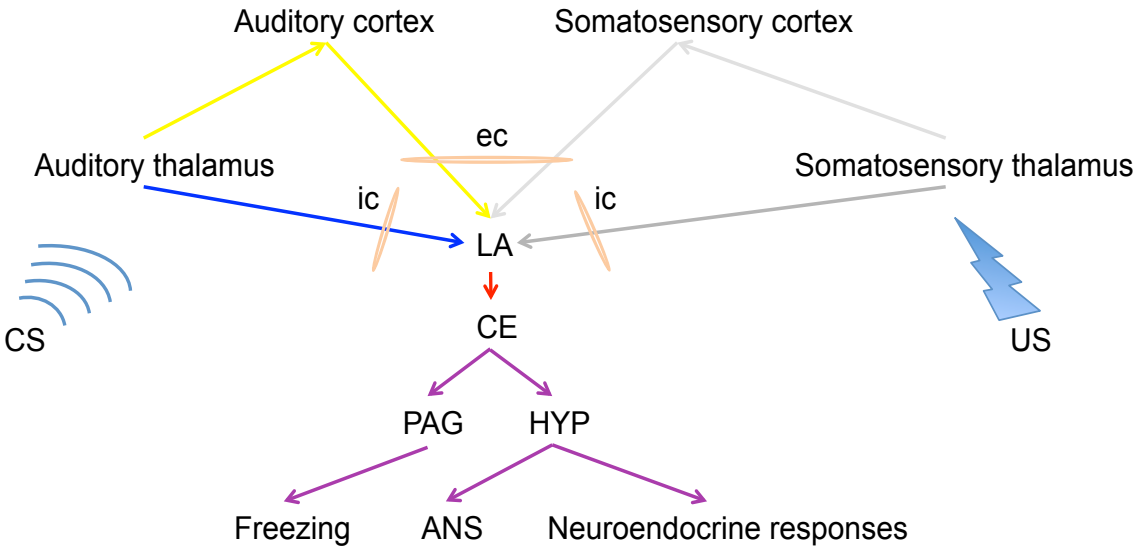

Figure 1. The principal brain regions involved in the processing of fear conditioning.

The sound emitted by the CS and the electrical foot pain induced by the US reach the auditory and somatosensory thalamic and cortical nuclei, respectively. The sensory thalamic input directly reaches the LA (blue arrow), whereas the cortical input to the LA is indirect since the sensory information passes first through the thalamus, which then projects to the sensory cortex (yellow arrows). The thalamic and cortical pathways send inputs through the internal and external capsule to the LA, from where the information is transmitted to the CE (red arrow) The CE outputs reach certain brain regions involved in eliciting fear responses (purple arrows), such as the PAG for freezing behavior, the autonomic nervous system (ANS) and the hypothalamus for releasing hormones responsible for fear reactions. The auditory pathway is better characterized than the somatosensory pathway (grey arrows).

3.3.2 The amygdala

The amygdala is the main brain region responsible for fear processing, fear learning and the storage of fear memory [1-3,5,7,8,14,19,21,23,25,26,34]. The morphology of the amygdala, as well as its role in fear memory, has been highly conserved across evolution [1,18,35,36].

3.3.2.1 The amygdala and its role in fear and PTSD

Brown and Schafer had hypothesized its involvement in the processing of fear responses as early as 1888 [37]. They observed that rhesus monkeys with a lesion in the temporal lobe that included the amygdala had social and emotional deficits [37]. In 1939, Klüver and Bucy repeated Brown and Schafer's experiments with better accuracy at targeting the amygdala, and showed that injured monkeys are hypo-emotional and do not manifest any fear upon aversive stimuli [38]. Similarly, humans suffering from Urbach-Wiethe disease, a rare genetic disorder that often leads to bilateral amygdala abnormalities, do not exhibit any fear responses [39] and have deficits in the recognition of fearful facial expressions [40]. Furthermore, electrical stimulation of the amygdala in humans results in autonomic reactions of fear or anxiety [41]. Additionally, in rodents, lesions of the amygdala prevent conditioning freezing [8,17,26-30,33,42-46]. Finally, selected rodents with a physiological deficit in fear extinction (physiological resistance to extinction of fear observed during classical fear conditioning paradigm) showed a dysfunction in amygdalar activity [47]. This abnormal amygdalar activity is also observed in humans suffering from PTSD, and is associated with an impaired fear extinction compared to healthy controls [3].

3.3.2.2 The anatomy and circuitry of the amygdala in fear memory

The amygdala belongs to the limbic system, an important area for emotional processing in both humans and rodents [48]. It is localized ventrolaterally in the temporal lobe, and is composed of several nuclei and subnuclei[49]. The most relevant nuclei of the amygdala related to fear are the lateral (LA), basolateral (BLA, which includes basal (B) and the accessory basal (AB)), and the central (CE) nuclei [49] (Figure 2).

The LA is the first amygdalar nucleus that receives the CS inputs from the auditory thalamus and auditory cortex [26]. Therefore, this nucleus is of crucial relevance in the processing of fear. Moreover, specific damage to the LA disrupts fear memory [29]. The LA integrates auditory information from thalamic and cortical inputs and forwards the sensory information via direct or indirect (via BLA) outputs to the CE (Figure 2). The LA contains three other sub-nuclei; the dorsal sub-nucleus (dLA) is the first site station receiving both thalamic and cortical afferents, the ventral (vLA) and medial (mLA) areas receive unidirectional direct inputs from the dLA. The vLA and mLA both forward the sensory information to both BLA and CE [49]. The CE then projects to the brain stem and the hypothalamus (HYP) for eliciting conditioned fear responses (CRs)[42]. Projections from the CE to the periaqueductal gray (PAG) lead to freezing and hypoalgesia, while projections to the lateral hypothalamus are involved in the control of conditioned cardiovascular responses [1,42] (Figure 1). Thus, the amygdala is situated between sensory systems involved in the processing of the conditioned fear stimuli and motor brain areas implicated in the control of the conditioned fear responses.

3.3.3 The role of the sensory thalamus in fear and PTSD

The thalamus is the principal relay center for processing information to the cortex [50]. Indeed, all sensory information, except for olfaction, is transmitted through the thalamus before reaching the cerebral cortex [51]. The neural activity within the thalamic nuclei is involved in attention and arousal [52]. Interestingly, both attention and arousal are clearly reduced in PTSD patients [3,53]. In fact, a common feature observed in PTSD is thalamic dysfunction, which can be detected by neuroimaging studies [53-57]. It has even been shown that this thalamic dysfunction may be the underlying mechanism responsible for flashbacks (intrusive memories of the traumatic event) in PTSD [57].

3.3.3.1 The anatomy and circuitry of the sensory thalamus in fear memory

In rodents, lesions of the auditory thalamic nuclei interfere with fear conditioning [58]. Retrograde labeling from the LA identified the specific nuclei involved in the transmission of the auditory CS[30,32]. These nuclei are those that receive afferents from the inferior colliculus, an obligatory relay center in the ascending auditory system. These nuclei are the ventral, the dorsal, and the medial part of the medial geniculate body (MGB, including MGv, MGd, and MGm), as well as areas of the posterior thalamus, including posterior intralaminar nuclei (PIN)[26,28,30,32,58]. The thalamo-dLA projection consists of a direct transmission from auditory processing regions in the thalamus, mostly from the MGm/PIN to the dLA (Figure 2).

3.3.4 The sensory cortex and its role in fear and PTSD

The sensory cortex plays an important role in hierarchizing the sensory processing, which starts with the primary sensory areas and continues in unimodal and multimodal association areas, as well as the prefrontal cortex [53]. These higher order sensory areas are involved in the integration of information coming from the primary sensory cortex and have a role in coordinating aspects of multimodal somatosensory-visual-auditory integration [53]. The greater neural activity in the amygdala observed by fMRI upon presentation of the CS in PTSD compared to healthy humans correlates with enhanced activity in the anterior cingulate cortex (ACC) and orbitofrontal cortex of the sensory cortex [5,59]. The activities of these cortices are interconnected and are mainly involved in decision-making [60,61], socially-driven interactions [62], and empathy-related responses [63], all of which are disrupted in PTSD [8-10,53,64].

3.3.4.1 The anatomy and circuitry of the sensory cortex in fear memory

In rodents, lesions and tracing studies led to identification of the main sensory cortical areas involved in the circuit of fear [27,65-67]. The nuclei of the sensory cortex involved in auditory fear conditioning are the primary auditory cortex (TE1), the secondary auditory cortex (TE2, TE3) and the perirhinal cortex (PRh) (Figure 2).

While the thalamo-dLA projection consists of a direct transmission from auditory processing regions in the thalamus to the amygdala, the cortico-dLA projection is an indirect transmission arising first from the auditory thalamic nuclei (MGv, MGd and MGm/PIN) to the TE1, followed by TE1 to TE2/TE3,

and finally from TE2/TE3 to PRh, ending eventually in the dLA[28]. Thus, within the thalamic nuclei, the MGv and MGd nuclei project to dLA exclusively via cortical relays, while the MGm/PIN nucleus projects both directly and indirectly to the LA [1] (Figure 2).

In summary, the fear-related emotional inputs arrive first to the dLA by the direct thalamic pathway as a rapid but coarse warning signal, whereas the indirect cortical pathway to the dLA provides a slower but more accurate representation of the same emotional stimulus. The cortical information can thus confirm the first and fast warning signal from the thalamus, thereby giving rise to CRs if the stimulus represents a real threat [1,48].

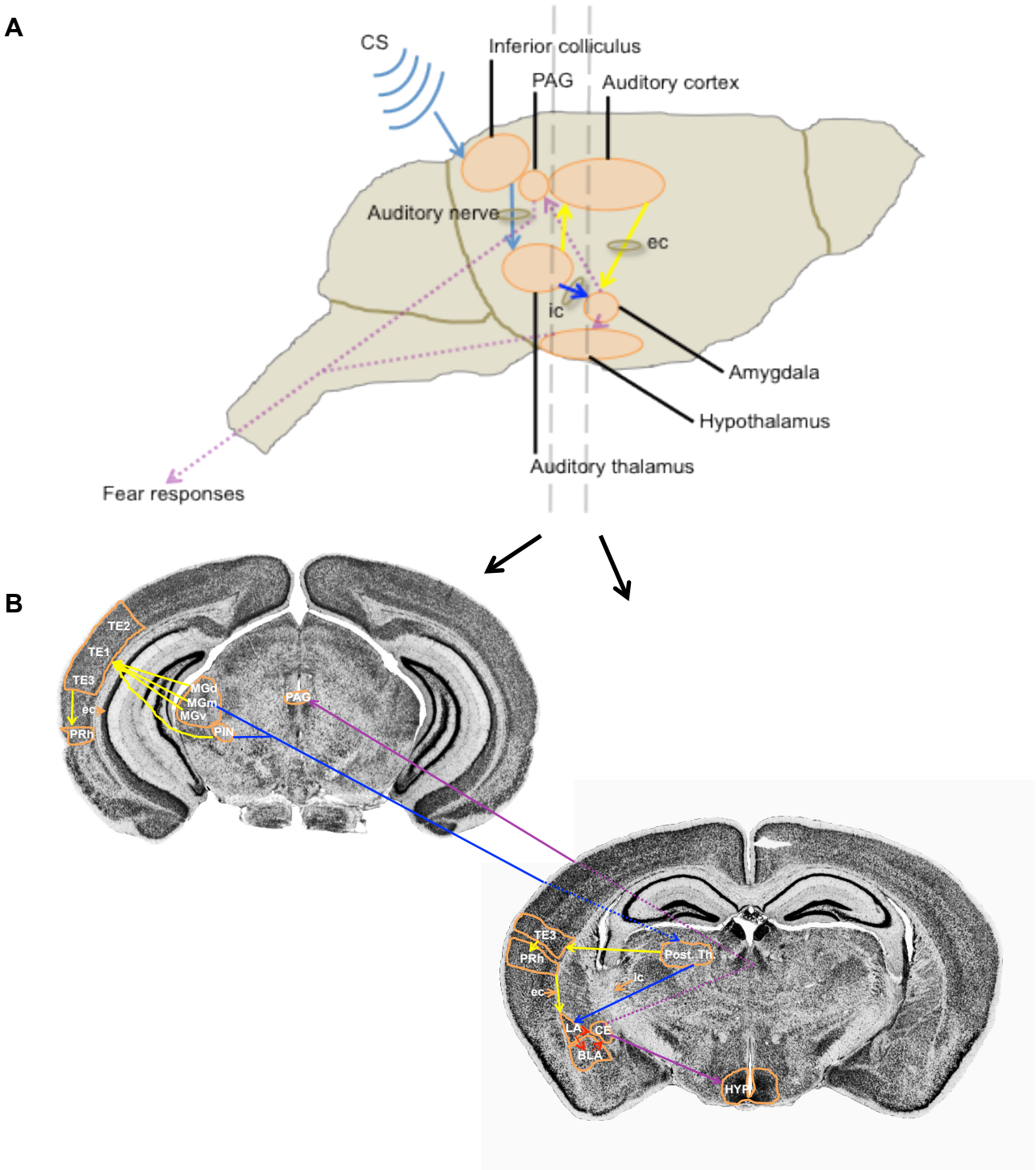

Figure 2. The auditory pathways involved in fear.

A, a schema of an adult rodent brain showing the CS-auditory pathways activated upon a tone emission. B, two coronal slices (taken from http://www.mbl.org) representing two sections (bregma -2.92 and 1.64 respectively) from posterior to more anterior (dashed grey lines in A). The CS-related information reaches the auditory thalamus via the inferior colliculus. The thalamus sends direct input (blue arrows) to the LA through the internal capsule (ic). The thalamic nuclei responsible for direct transmissions to the LA are the MGm and PIN (Down, left coronal brain slice). The cortico-LA connection is an indirect transmission from thalamic nuclei such as MGd and MGv (Yellow arrows). The primary auditory cortex, TE1, after receiving thalamic projections, forwards the sensory information to the secondary auditory cortical areas (TE2 and TE3) and the PRh, which finally project afferents to the LA through the external capsule (ec). The LA integrates the CS-related information and gives the information to the CE either directly or indirectly (red arrows) via the BLA. The CE is responsible for fear responses by activating several brain regions such as the HYP and the PAG (purple arrows).

3.3.5 The anatomy and circuitry of fear extinction

Compared with the anatomy and circuitry of fear memory, much less is known about the underlying mechanisms of fear extinction, despite its potential clinical significance for the treatment of anxiety disorders, such as PTSD.

Indeed, an approach commonly used for the treatment of anxiety disorders is similar to fear extinction. In this extinction-based exposure therapy, the subject is repeatedly presented with the object or the situation related to their traumatic fear (CS) in the absence of the danger (US).

Thus, it is widely believed that there is a crucial need to understand the anatomy and circuitry of fear extinction, since it might lead to improvements in the treatment of anxiety disorders.

Based on the fear conditioning paradigm, two distinct hypothesis were brought to explain the underlying anatomical and molecular mechanisms of fear extinction: 1) a new inhibitory memory that competes with the initial fear memory for the control of the expression of fear responses, or/and 2) a weakening of the initial CS-US association leading to fear erasure [3,21,47].

3.3.5.1 Fear extinction: a new inhibitory memory?

This first hypothesis is based on the fact that extinction-based exposure therapy does not erase or reverse the initial fear memory, since the conditioned fear responses can reappear with the passage of time (spontaneous recovery), if the CS is presented in a different context than where the exposure therapy took place (fear renewal), or if unexpected USs occur in the context of the exposure therapy but prior to testing extinction recall (fear reinstatement). Therefore, these observations suggest that the

behavioral properties of fear extinction depend on the development of a new inhibitory memory competing with the initial conditioned fear for the control of the fear responses. Studies in both rodents and humans elucidated the brain anatomy and circuitry involved in this statement of fear extinction [3,21,47].

3.3.5.1.1 The anatomy and circuitry of fear extinction: new inhibitory memory

Fear extinction associated with lesion and electrophysiological experiments led to the interconnected brain regions that have been implicated in this form of fear extinction, which are the vLA and BLA nuclei of the amygdala, the infralimbic (IL) and prelimbic (PL) regions of the medial prefrontal cortex (mPFC), and the hippocampus [8,21,68,69]. The studies in rodents and humans related to the anatomy and circuitry of fear extinction as a new inhibitory learning mechanism shared homologous brain regions and similar connections between them [68].

The PL in rodents and the dorsal anterior cingulate cortex (dACC) in humans are homologous. They both project to the vLA and the BLA, leading to the expression of the conditioned fear responses. The IL in rodents and the ventro-medial PFC (vmPFC) in humans are homologous. They both project to the BLA and to the intercalated cells surrounding the amygdala (ITC; a group of cells using gamma-aminobutyric acid (GABA) as the main inhibitory neurotransmitter, with inhibitory influence on the CE output neurons), leading to the weakening of the conditioned fear responses [21,69]. Indeed, it has consistently been demonstrated that extinction performed in healthy humans is associated with an increased activity in the vmPFC and a decreased activity in the dACC [70]. In contrast, there is evidence showing that human subjects suffering of PTSD exhibit a reduced activity in the vmPFC, and an increased

activity of the dACC during extinction compared to healthy subjects [71]. This suggests that dACC hyperactivity and vmPFC hypoactivity may contribute to the fear extinction impairment observed in PTSD [69].

The hippocampus is activated during extinction to transmit contextual sensory information to the mPFC in both rodents and humans. It is proposed that when the CS is presented in the same context than where extinction training occurred, the hippocampus sends glutamatergic (glutamate is the main excitatory neurotransmitter in the central nervous system) projections to the IL region, thus activating it. The IL in turn activates inhibitory interneurons in the BLA that inhibit the output neurons in the CE, therefore preventing conditioned fear responses [47,69]. However, when the CS is induced in a different context than where the extinction training happened, the hippocampus does not activate the IL cortex. Then the CE activity is not inhibited and the conditioned fear responses return [69]. Moreover, fear extinction increases hippocampal and vmPFC activity in the extinction context, but not in the original conditioning context [69].

To summarize, fear extinction, as a new inhibitory memory, relies on the amygdala (mostly the BLA and at a less extent vLA) for the storage of new inhibitory extinction memory, the hippocampus for the processing of the contextual information, and the IL for the consolidation and retrieval of extinction memory.

3.3.5.2 Fear extinction: erasure of the previously acquired fear memory?

There is evidence that the previously acquired conditioned fear memory is reversed by extinction, triggering fear erasure. The underlying brain anatomy

and network of fear erasure involve the same brain regions than the one activated during fear memory acquisition [72-76]. Thus, as in fear memory, during fear erasure the dLA receives sensory inputs from the auditory thalamic (mMGB/PIN) and cortical nuclei (TE1 to TE3 and Prh).

It is important to note that these two models of fear extinction may co-exist. The new inhibitory memory mostly involves the BLA and the vLA, whereas the fear erasure model implicates the dLA [21]. Since the dLA sends unidirectional projections to the vLA and that it is the first sub-nucleus of the amygdala that integrates fear-related sensory information from thalamic and cortical projections, it is of crucial relevance to elucidate the molecular mechanisms occurring in this amygdalar sub-nucleus during fear erasure.

3.3.5.2.1 Distinct contribution of thalamic and cortical inputs to the dLA in fear memory

As described above (2.3.1: the anatomy and circuitry of fear memory), during fear memory acquisition, the thalamic and cortical afferents send convergent but temporally separated inputs to the dLA [26,27], with the thalamic input to the dLA arriving about 12 ms faster than the cortical input[27,28]. Humeau and colleagues (2005) found that these convergent inputs reach a single cell in the dLA by targeting the same dendrite but contacting neighboring and morphologically distinct types of spines [77]. Larger spines are contacted by thalamic afferents, whereas smaller spines are contacted by cortical afferents [77]. This suggests that each pathway may carry different sensory information to the LA neurons [77,78]. Nevertheless, both inputs to the dLA are sufficient to support fear conditioning as well as fear extinction [28], although by different mechanisms. For example, the direct inputs from the auditory thalamus to the

LA seem to have a larger processing capacity in auditory fear conditioning elicited by a simple (classical) auditory CS than cortical inputs [27,78]. On the other hand, the indirect afferents from the auditory cortex to the LA contribute more to the processing of a complex CS [79-81], for example, in a differential (and non-classical) fear conditioning paradigm. In the differential fear-conditioning paradigm, the tested animal must discriminate between a CS paired with an US and an unpaired CS alone (which does not result in a negative expectation)[79-81].

The transmission of the CS-related information from both afferents to the dLA is induced by the ability of these inputs to change the strength of synaptic transmission during fear memory and its extinction. The ability of the synapses to change in strength between two neurons in response to sensory signals, such as for example those elicited by the CS, is known as synaptic plasticity. Synaptic plasticity is believed to be the basis of information storage in the brain. Since in the dLA the synaptic strength responsible for the acquisition of fear memory is reversed during the erasure of fear memory model of fear extinction, studying synaptic plasticity in the dLA, especially during fear erasure, may result in novel understandings of how to ameliorate this behavioral deficit in PTSD.

3.4 Synaptic plasticity underlying fear erasure in the dLA

The two most widely studied forms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD)[82,83]. LTP is characterized by a persistent long-lasting enhancement of synaptic strength, whereas LTD is defined as a long-lasting reduction in synaptic strength[83,84]. Following the

acquisition of fear memory, both inputs to the LA show an enhanced synaptic transmission. On the other hand, previously enhanced transmission at both thalamo- and cortico- dLA synapses is reversed during extinction of fear[72-74,85]. Therefore, thalamic and cortical projections undergo opposite changes in synaptic strength during fear conditioning and fear extinction [73,74,86-93].

Extensive studies have shown that long-lasting enhancement in synaptic transmission, such as those occurring in LTP at both thalamo- and cortico-dLA synapses, may be the underlying mechanism mediating associative memory of the CS-US association during auditory fear conditioning [72,89,90,92,94,95]. On the other hand, LTD is thought to be the synaptic mechanism of fear extinction, since a long-lasting depression was observed at both thalamic and cortical inputs to the LA during fear extinction [73,74]. Additionally, *in vivo* LTD induction after fear conditioning performed either in rats [96] or primates [97] gives rise to fear extinction. Given that a defect in fear extinction is thought to contribute to PTSD, studying the molecular mechanisms underlying LTD associated with fear extinction is of vital importance to the development of future treatments for PTSD [84].

3.4.1 Molecular mechanisms underlying LTD in the dLA

Both thalamic and cortical synaptic connections to the pyramidal dLA neurons are glutamatergic [29,31,98], meaning that they use glutamate as the main neurotransmitter. Furthermore, the thalamo- and cortico-LA synapses are asymmetric [98], which is often associated with excitatory neurotransmission [99,100]. Moreover, both thalamic and cortical inputs to pyramidal LA neurons contain glutamatergic receptors, such as the α-amino-3-hydroxy-5-methyl-4-

isoxazolepropionic acid receptor (AMPAR) and N-methyl-D-aspartate receptor (NMDAR)[101-104]. These two receptors are crucial for NMDAR-dependent synaptic plasticity, the main form of plasticity in the brain [105,106].

In fact, NMDAR-dependent LTD is the most common type of LTD within the central nervous system [84]. For example, perfusion of D-APV, a strong and potent NMDAR antagonist, abolishes LTD in many different brain regions [84]. In the LA, NMDAR inhibition abolishes both LTD [84,107-111] and fear extinction [84,112,113]. Additionally, NMDAR-dependent LTD is occluded at thalamo- and cortico-LA synapses in slices from extinction-trained rats [73,74]. Thus, a strong link between NMDAR-dependent LTD and extinction has been demonstrated. However, the molecular mechanisms underlying NMDAR-dependent LTD in the LA at both thalamic and cortical inputs remain poorly understood.

Since pharmacologically blocking NMDAR activity blocks LTD as well as fear extinction, drug therapies aimed at activating NMDARs, by injection of D-cycloserin (DCS), a partial NMDAR-agonist, in patients suffering from PTSD is commonly used as a treatment in association with extinction-based psychotherapy training (see discussion, [8,20,114]). However to date, this type of pharmacotherapy has a very low success rate due to side effects, such as epilepsy and memory loss. This may be caused by the ubiquitous need for NMDARs either in LTP or in LTD and other types of plasticity. Therefore, a promising approach for improving these pharmacotherapies in PTSD would be to investigate the downstream molecular pathways underlying the regulation of the NMDARs at both inputs to the dLA. This may help to decrease the side effects after therapy, since both LTP and LTD require distinct downstream regulative pathways but share NMDAR requirements.

3.4.2 The NMDAR in LTD

NMDARs are ionotropic transmembrane receptors for glutamate, and known to be the most important trigger for synaptic plasticity [106]. They are ligand- and voltage- dependent, as they are activated mostly by the removal of the Mg^{2+} blockade upon depolarization, and require glycine and glutamate binding for channel opening [115]. Once the ion channel is open, the NMDAR enables Na^{+} and Ca^{2+} ions to enter, and K^{+} to exit the cell [116], thereby leading to activation of Ca^{2+}- dependent intracellular signaling pathways. It is thought that a strong depolarization gives rise to a high postsynaptic Ca^{2+} influx, which leads to LTP, whereas a weak depolarization gives rise to a low or moderate postsynaptic Ca^{2+} influx, which results in LTD [84,117]. Additionally, the Ca^{2+}-mediated intracellular signaling pathways downstream of the NMDARs can recruit different molecular targets depending on the NMDAR subunit composition [116].

In the brain, the NMDARs are mostly constituted of two NR1 subunits associated with two NR2 subunits, where NR1 contains the glycine sites and NR2 gathers glutamate, thus conferring the electrophysiological properties of the receptor [115,116,118-120]. Among the four NR2 subunits (NR2A-NR2D), NR2A and NR2B are the most extensively studied, due to their broad expression in the brain [119], and because they have been shown to have important roles in synaptic plasticity [115,116,121,122]. For example, in cortical layers, in the CA1 of the hippocampus and at thalamo-LA synapses of the amygdala, NR2A-containing NMDARs are required for LTP induction, whereas the induction of LTD recruits specifically NR2B-containing NMDARs [121-124]. Although less studied due to lack of specific and efficient antibodies, NR2C and NR2D

subunits seem to play a more important role in LTD compared to LTP, as demonstrated in the hippocampal CA1 subregion [125].

It is thought that thalamic and cortical pathways carry distinct sensory information to the LA, and the difference between these inputs is hypothesized to be due to divergence in their NR2 subunit specificity [105,126]. However, whether NMDAR-dependent LTD recruits distinct types of NR2 subunits-containing NMDARs at both thalamic and cortical inputs to the LA remains unclear [127,128]. This is relevant to explore since the distinct types of NR2 subunits composing the receptor may also attribute a difference in synaptic localization [105,126]. For example, the NR2C/D-containing NMDARs may be activated even in the absence of depolarization or only by glutamate binding [126] due to their electrophysiological properties. Thus, they are mostly present at the presynaptic compartment of a neuron [129], in contrast to NR2A/B-containing NMDARs, which are mostly postsynaptically expressed and require both depolarization and glutamate binding[106,115,116,119,126,130-132]. Indeed, in the neocortex, presynaptic NR2C/D-containing NMDARs are believed to be involved in presynaptic LTD[133,134], and at thalamo-LA synapses NR2B subunits are exclusively expressed postsynaptically[130]. However, NR2C/D-containing NMDARs have never been explored in the amygdalar LTD. A difference in NR2 subunits and in the synaptic localization of the NMDARs may prove that both afferents carry distinct CS-related sensory information to the LA due to their distinct NR2 subunits. Therefore, further investigation of the contribution of distinct NR2 subunits during LTD at both afferents to the LA will provide insight into whether and how these pathways are differentially regulated during LTD and thus fear extinction.

The regulation of the NMDAR-dependent LTD at both inputs to the dLA remains still poorly known. However, this type of LTD has been extensively studied in the hippocampus. Thus, before going into details about the molecular mechanisms underlying LTD in the dLA, below an overview of the regulation of the NMDAR-dependent LTD in the hippocampus.

3.4.3 Protein-phosphatase 1 and its role in the regulation of hippocampal NMDAR-dependent LTD

NMDAR-dependent LTD is mainly regulated by post-translational modifications, such as dephosphorylation, by signaling molecules regulating synaptic transmission [135-146]. Protein-phosphatase 1 (PP1), a member of the serine (Ser) and threonine (Thr) family of protein phosphatases (PPs), is one of the most highly conserved proteins in eukaryotic cells[136,137,147-149], and is responsible for regulating a variety of cellular processes[150-152]. The role of PP1 has mostly been analyzed in the hippocampus, where it regulates learning and memory[146] through its action on RNA splicing[153], gene transcription[141,142], apoptosis[154], and synaptic transmission[155-157]. Specifically, modulation of PP1 activity influences the directionality of synaptic plasticity. Whereas PP1 inhibition is required for LTP[158-160], PP1 activation leads to LTD[155-157,160].

Amongst the PP family members, PP1, PP2A, PP2B (also known as calcineurin), PP4, PP5, PP6 and PP7 are expressed in mammalian neurons. In particular, PP1, PP2A and PP2B account for the majority of Ser and Thr PP activity *in vivo*[150-152]. Together, they dephosphorylate more than 90% of the neuronal phosphate-bound proteins[161]. Due to its ubiquitous localization and

its function in regulating a broad range of cellular functions, PP1 is the best characterized of the PPs[149].

Thus, when low frequency stimulation (LFS) is applied to CA3-CA1 Schaeffer collaterals of the hippocampus, the influx of Ca^{2+} entering through the NMDARs into the postsynaptic CA1 neurons binds to calmodulin (CaM), leading to the activation of a protein-phosphatase (PP) cascade. Once activated, the calcium/calmodulin-dependent PP2B dephosphorylates inhibitor-1 (I1, a specific inhibitor of PP1), thereby releasing PP1 from I1 inhibition[156,157] (Figure 3).

Once PP1 is active, it is brought to the vicinity of the NMDARs found in the postsynaptic density (PSD), by scaffolding proteins[155,162,163]. Its role during LTD consists of dephosphorylating various targets[84] involved in synaptic plasticity, including AMPAR[162-164] and NMDAR[131,165]. The regulation of AMPAR and NMDAR plays a crucial role in hippocampal LTD [84,106,166]. For example, the PP1-mediated dephosphorylation of a specific serine residue on the AMPAR during LTD is responsible for receptor internalization and is known to enable LTD expression [110,132,162-164,166]. On the other hand, whether PP1 dephosphorylates NMDARs during LTD is still unclear. In the hippocampus, the NMDARs showed decreased activity during NMDAR-dependent LTD[165]. It is hypothesized that PP1 is responsible for this change in NMDARs activity [165] (Figure 3).

As a key regulator of NMDAR-dependent LTD in the hippocampus, PP1 has been shown to play a crucial role in cognitive functions. In fact, activation of PP1 limits hippocampus-dependent learning and memory and promotes forgetting [146], whereas PP1 inhibition enhances learning and memory [140,146].

Notably, LTP impairment is rescued by PP1 inhibition in a mouse model of Alzheimer's disease [144]. In addition, our lab recently demonstrated that the role of PP1 might extend beyond the hippocampus to include the amygdala [140], where an enhanced fear memory upon genetic PP1 inhibition has been associated with an improved LTP at cortico-LA synapses[140].

Given that PP1 is essential to LTD in the hippocampus, and that LTD is the synaptic correlate of fear extinction in the amygdala, it is plausible to hypothesize that PP1 may regulate fear extinction through modulating LTD in the amygdala. If this were true, it would be possible that a novel treatment for PTSD could involve enhancing LTD by increasing PP1 activity in the amygdala of individuals by facilitating fear extinction. However, the role of PP1 in amygdalar LTD remains unexplored. The following chapter provides an overview of the current knowledge related to LTD at thalamic and cortical inputs to the dLA is provided.

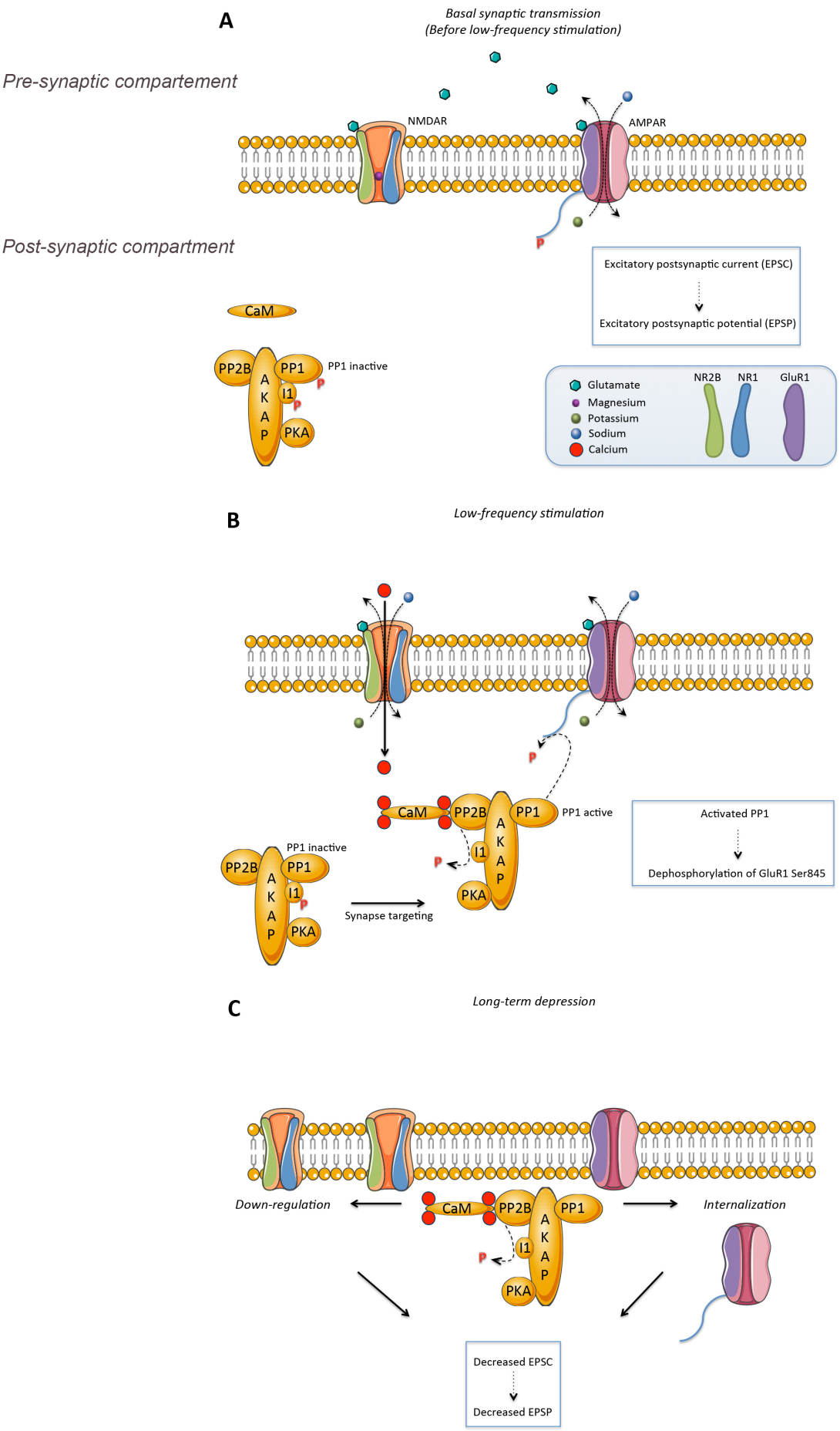
A
Basal synaptic transmission
(Before low-frequency stimulation)
Pre-synaptic compartement
NMDAR
AMPAR
Post-synaptic compartment
Excitatory postsynaptic current (EPSC)
Excitatory postsynaptic potential (EPSP)
CaM
PP2B
PP1
PP1 inactive
AKAP
I1
PKA
Glutamate
Magnesium
Potassium
Sodium
Calcium
NR2B
NR1
GluR1
B
Low-frequency stimulation
CaM
PP2B
PP1
PP1 active
AKAP
I1
PKA
Activated PP1
Dephosphorylation of GluR1 Ser845
PP1 inactive
Synapse targeting
C
Long-term depression
Down-regulation
Internalization
Decreased EPSC
Decreased EPSP

Figure 3. The role of PP1 in hippocampal post-synaptic NMDAR-dependent LTD.

A, in the basal state, the synaptic transmission is mediated by the flux of sodium (Na^{2+}) into the cell and the flux of potassium (K^{+}) out from the internal compartment of the neuron through the AMPAR. This creates a excitatory post-synaptic current (EPSC) changing the membrane potential (EPSP). PP1 is not active in basal synaptic transmission, since I1 inhibits its activity. PP2B is Ca^{2+}- dependent and therefore at basal synaptic transmission, PP2B is inactive, since there is no calcium (Ca^{2+}) influx into the cell through NMDAR. The NMDAR is not open yet because of the Mg^{2+} blockade. I1 is activated upon phosphorylation mediated by PKA. PKA, I1, PP2B and PP1 are gathered by A-kinase anchoring protein (AKAP). **B**, upon LFS, NMDAR activation triggers a low influx of Ca^{2+} into the postsynaptic neuronal compartment. The activation of the NMDAR leads to the transport of PP2B and PP1 by anchoring proteins such as AKAP, which binds PP1, PP2B and PKA. The calmodulin (CaM) detects Ca^{2+} and leads to the activation of the Ca^{2+}-dependent PP2B. Once activated, PP2B dephosphorylates I1, releasing PP1 blockade and leading to its activation. **C**, amongst the various targets that PP1 can dephosphorylate, the GluR1 subunit of the AMPAR at its Ser845 is of crucial importance for NMDAR-dependent LTD, since its dephosphorylation leads to the internalization of the receptor. PP1 is also able to act on the NMDAR by an unknown mechanism leading to the down-regulation of the receptor. The drawings of the cell membranes and the receptors have been taken from http://www.servier.com/Powerpoint-image-bank.

3.4.4 LTD at the thalamic pathway

At the thalamic fibers to the LA, LTD is input-specific and postsynaptic[107]. Its induction is most likely mediated by postsynaptic NR2B-containing NMDARs[121,128], and NR2B activation leads to endocytosis of AMPARs for LTD expression[110,132]. This process is made possible by inhibition of protein kinase Mζ (PKMζ), which is known to be essential for the formation of long-term memories by blocking endocytosis of AMPARs[167].

Interestingly, blocking NR2B-containing NMDARs as well as blocking endocytosis of AMPARs impairs the extinction, but not the expression, of conditioned fear[121,168]. Additionally, LTD induced at thalamic inputs to the LA in brain slices harvested from fear conditioned rats depends on NR2B-containing NMDARs[74,76] and correlates with a decrease in AMPARs localized to synaptosomes in the LA[74]. This suggests that depotentiation and LTD may share the same molecular mechanisms at thalamo-LA synapses[84]. In addition, LTD involves mitogen activated protein kinase (MAPK) signaling[75], which is likely activated upon Ca^{2+} influx through L-type voltage dependent calcium channels (L-VDCCs) located postsynaptically[169]. Similar to NR2B-containing NMDARs, L-VDCCs are thought to be required specifically for fear extinction[170].

Furthermore, LTD not only involves NMDARs, but also the group I metabotropic glutamate receptors (mGluRs) for their induction[74], as well as an unspecified PP signaling cascade[107]. Indeed, non-specific blockade of PP2A/PP1 in the LA by perfusion of okaidic acid or calyculin A abolishes LTD at the thalamic pathway[107]. The existence of PPs at thalamo-LA synapses is consistent with a hypothetical role for PPs in endocytosis of AMPARs at

thalamo-dLA synapses, possibly triggered by activation of NMDARs[110,132]. However, whether and how PP1 is specifically required for thalamo-dLA LTD is still unknown (Figure 4).

3.4.5 LTD at the cortical pathway

Since LFS is unable to induce LTD at cortical naïve synapses, most of the electrophysiological work performed at cortico-dLA synapses makes use of depotentiation for studying cortical LTD. In this configuration, LFS succeeded in inducing LTD after LTP induction. This form of LTD, which is thought to share common downstream molecular pathways with *de novo* LTD, also depends on NMDARs[73,109], but surprisingly does not lead to AMPAR endocytosis[73]. In addition, cortico-LA LTD not only depends on NMDARs but also requires the activation of group II mGluRs[73]. This suggests LTD expression occurs presynaptically, since 1) group II mGluRs are mainly localized at the presynapses, at least in the hippocampal mossy fibers-CA3[171,172] and at the nucleus accumbens synapses[173], and 2) the internalization of AMPARs, which are exclusively postsynaptic, is not required[73]. However, group II mGluRs can occur both pre- and post-synaptically in several brain regions such as in the hippocampus and cortex[174]. Thus, whether cortical LTD is pre- or post-synaptic remains to be determined by additional experiments, for example by chelating postsynaptic Ca^{2+}, or specifically blocking activated postsynaptic NMDARs before LTD induction. LTD at the cortical pathway is also thought to recruit the NR2B subunit[128]. This finding remains controversial, because distinct NMDAR subtypes are believed to be expressed at thalamic and cortical inputs when

electrophysiological analyses were performed at basal synaptic transmission (no synaptic plasticity)[127]. Furthermore, electrically induced depotentiation *in vivo* reduces fear conditioning-mediated phosphorylation of MAPK and protein kinase B (PKB), which is correlated with increased activity of PP2B, and PP2B inhibition blocks fear extinction and MAPK dephosphorylation[109,175] (Figure 4). As PP2B seems to be involved in cortico-dLA depotentiation, PP1 may also play a role in this pathway during LTD. However, this potential role remains unexplored.

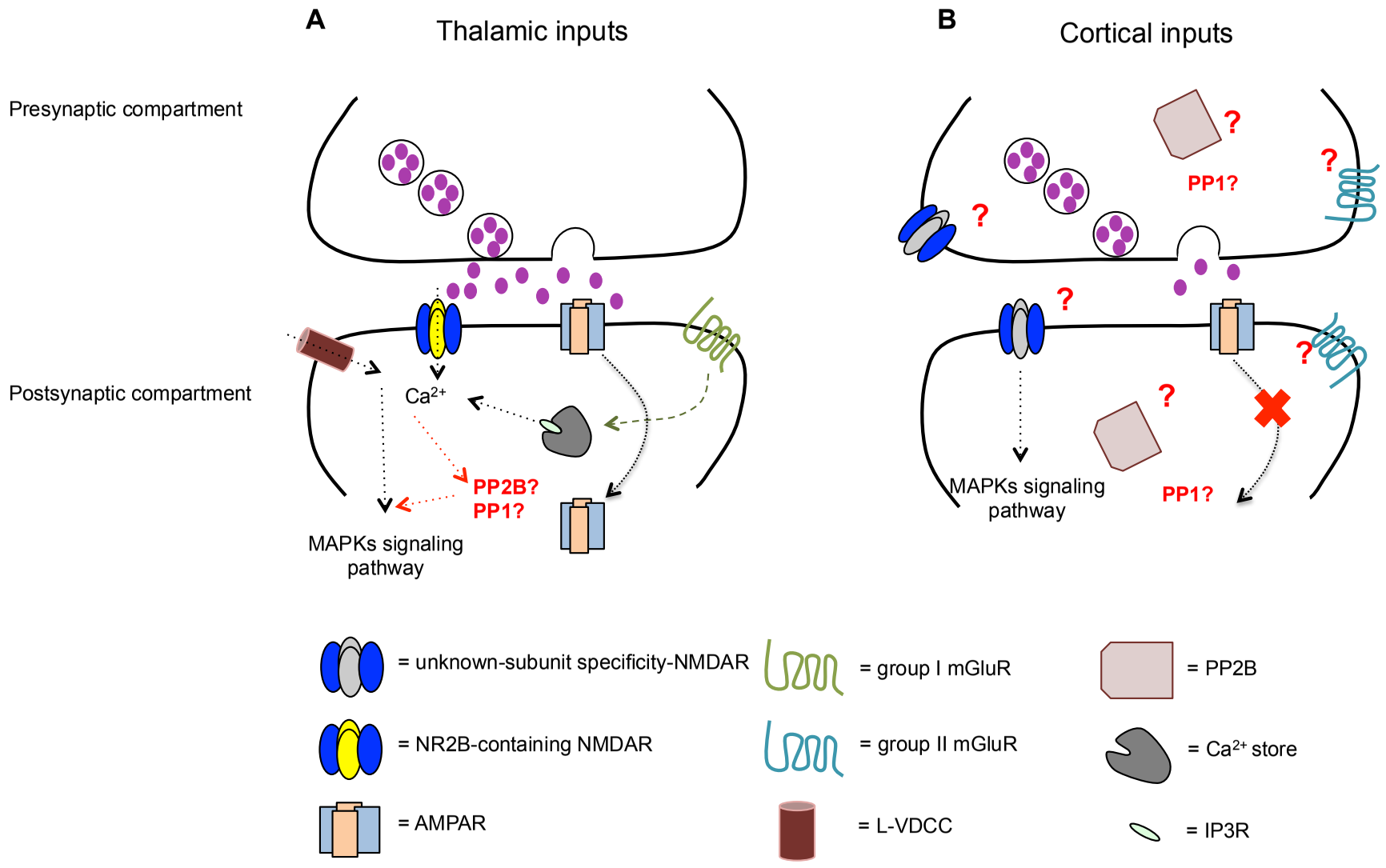
A
Thalamic inputs
B
Cortical inputs
Presynaptic compartment
Postsynaptic compartment
Ca2+
PP2B?
PP1?
MAPKs signaling pathway
?
PP1?
MAPKs signaling pathway
PP1?
= unknown-subunit specificity-NMDAR
= NR2B-containing NMDAR
= AMPAR
= group I mGluR
= group II mGluR
= L-VDCC
= PP2B
= Ca2+ store
= IP3R

Figure 4. The current knowledge of the molecular mechanisms underlying LTD at thalamic (left) and cortical (right) inputs to the LA neurons.

A, LTD at the thalamic inputs to the LA neurons is purely postsynaptic, requires NR2B-dependent NMDARs, which once activated trigger AMPAR endocytosis. L-VDCCs and group I mGluRs are also involved in LTD at thalamic afferents, both localized postsynaptically, where in collaboration with the NMDARs, they contribute to the postsynaptic rise of Ca^{2+}, leading to a downstream MAPKs signaling pathway. However, the postsynaptic PP2B/PP1 signaling cascade upon a rise of postsynaptic Ca^{2+} has not been elucidated yet. B, LTD at the cortical afferents is still poorly known. It implicates NMDARs and group II mGluRs. The subunit-specificity of the NMDARs at the cortical inputs needs to be confirmed, since it is still a debated topic. NMDARs activation leads also at these synapses to a downstream MAPKs signaling pathway. It has been suggested that LTD at the cortico-LA synapses depends on presynaptic mechanisms, since group II mGluRs are mostly localized at the presynapses. However in the hippocampal DG as well as other cortical areas, postsynaptic group II mGluRs are involved in LTD. Therefore the site of LTD at cortico-LA synapses remains unresolved. PP2B is needed in cortico-LA LTD, however it is still unknown whether PP1 is required.

4 Main focus of the thesis

In summary, a shared feature of thalamo- and cortico- dLA LTD is that they both require NMDARs. Despite the crucial role LTD plays in extinction of fear memory, the molecular mechanisms underlying LTD in the dLA remains poorly explored. The focus of my thesis was to further identify the molecular mechanisms underlying LTD at both thalamic and cortical inputs to the dLA, with a special focus on PP1 and the NMDAR subunits. The main questions explored in the presented experiments were 1) whether PP1 is required during NMDAR-dependent LTD, 2) which NR2 subunits are responsible for PP1 downstream activity, and 3) whether the site of LTD expression at both inputs to the LA is pre- or postsynaptic. The answers to these questions may provide new and crucial insights into how a potential treatment for anxiety disorders, such as PTSD, could be developed by targeting LTD-related signaling during extinction.

5 Main project

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Distinct molecular components for thalamic- and cortical-dependent plasticity in the lateral amygdala

Osvaldo Mirante[1, 2], Federico Brandalise[1], Johannes Bohacek[1, 2], and Isabelle M. Mansuy[1, 2, $]

1. Brain Research Institute, Medical Faculty of the University Zürich and

2. Department of Health Science and Technology of the Swiss Federal Institute of Technology, CH-8057 Zürich, Switzerland

Attribution of data: I have performed all experiments in this chapter. Federico brandalise strongly contributed to the whole-cell patch recordings and Dr. Johannes Bohacek strongly contributed to the writing of the publication.

Abstract

N-methyl-D-aspartate receptor (NMDAR)-dependent long-term depression (LTD) in the lateral nucleus of the amygdala (LA) is a form of synaptic plasticity thought to be a cellular substrate for the extinction of fear memory. The LA receives converging inputs from the sensory thalamus and neocortex that are weakened following fear extinction. Combining field and patch-clamp electrophysiological recordings in mice, we show that a paired-pulse low-frequency stimulation can induce a robust LTD at thalamic and cortical inputs to LA, and we identify different underlying molecular components at these pathways. We show that while LTD depends on NMDARs and activation of the protein phosphatases PP2B and PP1 at both pathways, it requires NR2B-containing NMDARs at the thalamic pathway, but NR2C/D-containing NMDARs at the cortical pathway. LTD appears to be induced postsynaptically at the thalamic input but presynaptically at the cortical input, since postsynaptic calcium chelation and NMDAR blockade prevent thalamic but not cortical LTD. These results highlight distinct molecular features of LTD in LA that may be relevant for traumatic memory and its erasure, and for pathologies such as post-traumatic stress disorder (PTSD).

5.1 Introduction

Synaptic plasticity, a property of neuronal connections characterized by a change in synaptic strength following neuron activation, is essential for memory formation but also for forgetting. Whether presynaptic stimulation increases or decreases synaptic strength depends on the magnitude of postsynaptic calcium elevation [176]. Long-term potentiation (LTP), a form of synaptic strengthening, is induced by a high rise in intracellular calcium concentration leading to activation of protein kinases. In contrast, long-term depression (LTD), a form of synaptic weakening, requires a moderate rise of intracellular calcium concentration that activates protein phosphatases including PP2B (calcineurin) and PP1 [156,157,160,177,178].

In the lateral amygdala (LA), LTP is associated with the formation of fear memory [89,90,95], while LTD is thought to underlie the extinction of fear memory [73,74,76]. Molecular manipulations that interfere with fear extinction do indeed impair LTD [108,109,121,168,179]. The LA is a complex limbic structure that integrates sensory information from cortical and thalamic afferents. These afferents are highly plastic [21,180] and converge onto single neurons in LA [77]. To date, LTD in LA has been mostly studied at the thalamic pathway, essentially because it is easier to induce than at the cortical pathway [107,169,181]. Similar to fear extinction [112,113,121,182], LTP at the thalamic pathway depends on NMDARs and is primarily associated with the NR2B subunit [111,113,121,128,132]. In contrast, the mechanisms of LTD at the cortical pathway remain unknown, but are postulated to be different from those at the thalamic pathway [183,184]. We investigated these mechanisms in adult mouse LA and examined whether they involve the phosphatases PP2B and PP1, and which NMDAR subunits

they recruit. Here we show that both PP2B and PP1 are involved in LTD in the amygdala, but that distinct NMDAR subunits are implicated at thalamic and cortical pathways. While LTD depends on NR2B-containing NMDARs at the thalamic pathway, it requires NR2C/D-containing NMDARs at the cortical pathway. We also show that LTD is induced postsynaptically at the thalamic pathway, but not at the cortical pathway.

5.2 Results

5.2.1 Pathway-specific LTD in LA

Using extracellular field recording, we first assessed whether a paired-pulse low frequency stimulation protocol (ppLFS) induces stable and input-specific LTD at thalamic and cortical afferents to the LA in slices from adult mouse (for electrode placement see Figure 1A). A robust LTD that lasted over 1hr was specifically induced at the pathway receiving ppLFS but not at a control pathway, both at thalamic (ppLFS pathway: 53.3 ± 4.3%, n = 18 vs. control pathway: 106.2 ± 10.9%, n = 11, $p < 0.001$, Figure 1B) and cortical input (ppLFS pathway: 52.7 ± 3.6%, n = 18 vs. control pathway: 108.6 ± 12.2%, n = 14, $p < 0.001$, Figure 1B). The magnitude of fEPSP suppression was comparable between thalamic and cortical ppLFS ($p > 0.9$). These results indicate that the ppLFS protocol leads to a strong and input-specific induction of LTD (LA-LTD) at both thalamic and cortical pathways to LA.

5.2.2 NMDAR-dependent LTD in LA depends on protein phosphatases

In the hippocampus, the most common form of LTD requires postsynaptic rise in calcium that depends on NMDARs, and is associated with activation of a

PP2B/PP1 signaling cascade [84]. Both PP2B and PP1 are known to be negative regulators of plasticity that further, can act as memory suppressors [146,185,186]. We thus first tested whether LA-LTD is NMDAR-dependent at both pathways using extracellular field recordings. LTD was fully blocked by the NMDAR antagonist D-APV (50 μM) at both, the thalamic (control: 50.6 ± 5.6%, n = 6; D-APV: 103.0 ± 10.4%, n = 9, $p < 0.001$, Figure 1C) and cortical (control: 53.7 ± 2.9%, n = 5; D-APV: 114.8 ± 9.9%, n = 9, $p < 0.001$, Figure 1C) pathway, demonstrating that LA-LTD depends on NMDARs at both pathways. Input/output (I/O) curves were not affected by D-APV, suggesting that basal synaptic transmission was not altered (Figure 1C, insets).

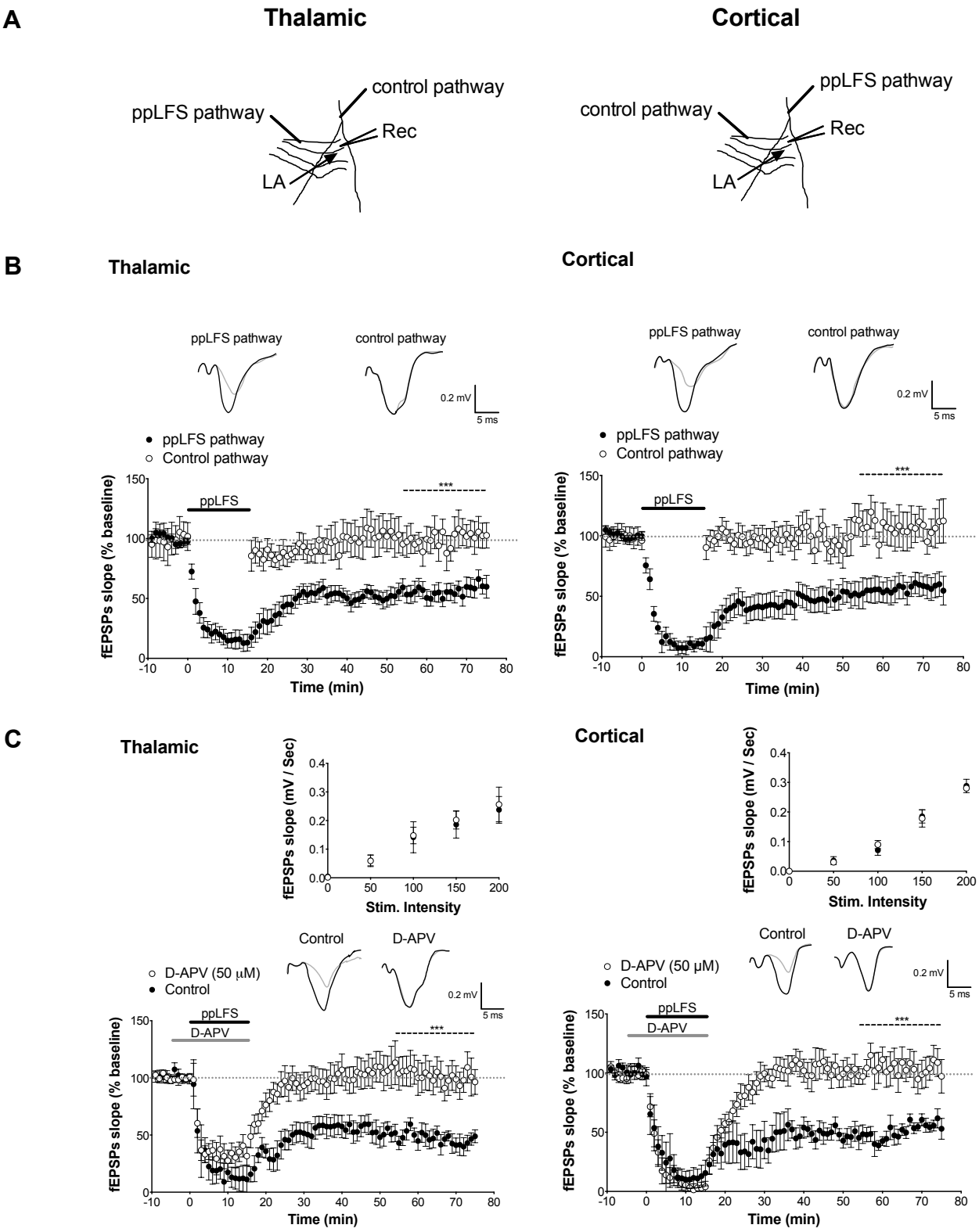
A
Thalamic
Cortical
ppLFS pathway
control pathway
Rec
LA
B
Thalamic
Cortical
ppLFS pathway
control pathway
0.2 mV
5 ms
ppLFS pathway
Control pathway
ppLFS
***
fEPSPs slope (% baseline)
Time (min)
C
Thalamic
Cortical
fEPSPs slope (mV / Sec)
Stim. Intensity
Control
D-APV
D-APV (50 μM)
Control
ppLFS
D-APV
fEPSPs slope (% baseline)
Time (min)

Figure 1. Input-specific NMDAR-dependent LTD at the thalamic and cortical pathways in the lateral amygdala.

(A) Schematic illustration of electrode placement for ppLFS and control pathway recording of the thalamic pathway (left) and the cortical pathway (right). (B) Robust, long-lasting LTD was specifically induced at the pathway receiving ppLFS (thalamic, n = 18; cortical, n = 18) but not at the control pathway (thalamic: n = 11; cortical: n = 14). Insets show representative traces of extracellular field potentials averaged across 10 mins before ppLFS (black line) and the last 10 minutes of recording after ppLFS (grey line). (C) D-APV (50 μM) prevents LTD at thalamic afferents (control: n = 6; D-APV: n = 9) and (E) at cortical afferents (control: n = 5; D-APV: n = 9). Insets show I/O curves on top and below representative traces of extracellular field potentials averaged across 10 mins before ppLFS (black line) and the last 10 minutes of recording after ppLFS (grey line). Data represent mean ± SEM. *** = $p < 0.001$.

Next, we examined whether PP2B and PP1 are involved in LA-LTD. Perfusion of the selective PP2B inhibitor FK-506 (100 μM) abolished LTD at both, thalamic (control: 54.9 ± 1.9%, n = 5; FK-506: 102.4 ± 10.9%, n = 5, $p < 0.01$, Figure 2A) and cortical (control: 48.3 ± 2.5%, n = 5; FK-506: 96.0 ± 5.0%, n = 5, $p < 0.001$, Figure 2A) pathways. Similarly, bath application of the specific PP1 inhibitor tautomycetin (4 nM) abolished LA-LTD at both pathways (Thalamic, control: 57.5 ± 5.2%, n = 5; tautomycetin: 101.4 ± 5.9%, n = 10, $p < 0.001$. Cortical, control: 52.4 ± 6.7%, n = 7; tautomycetin: 118.2 ± 20.8%, n = 8, $p < 0.05$, Figure 2B). I/O curves were not affected by FK-506 (Figure 2A, insets) or tautomycetin (Figure 2B, insets), suggesting that basal synaptic transmission was not altered. These results show that LA-LTD requires PP2B and PP1 at both thalamic and cortical pathways.

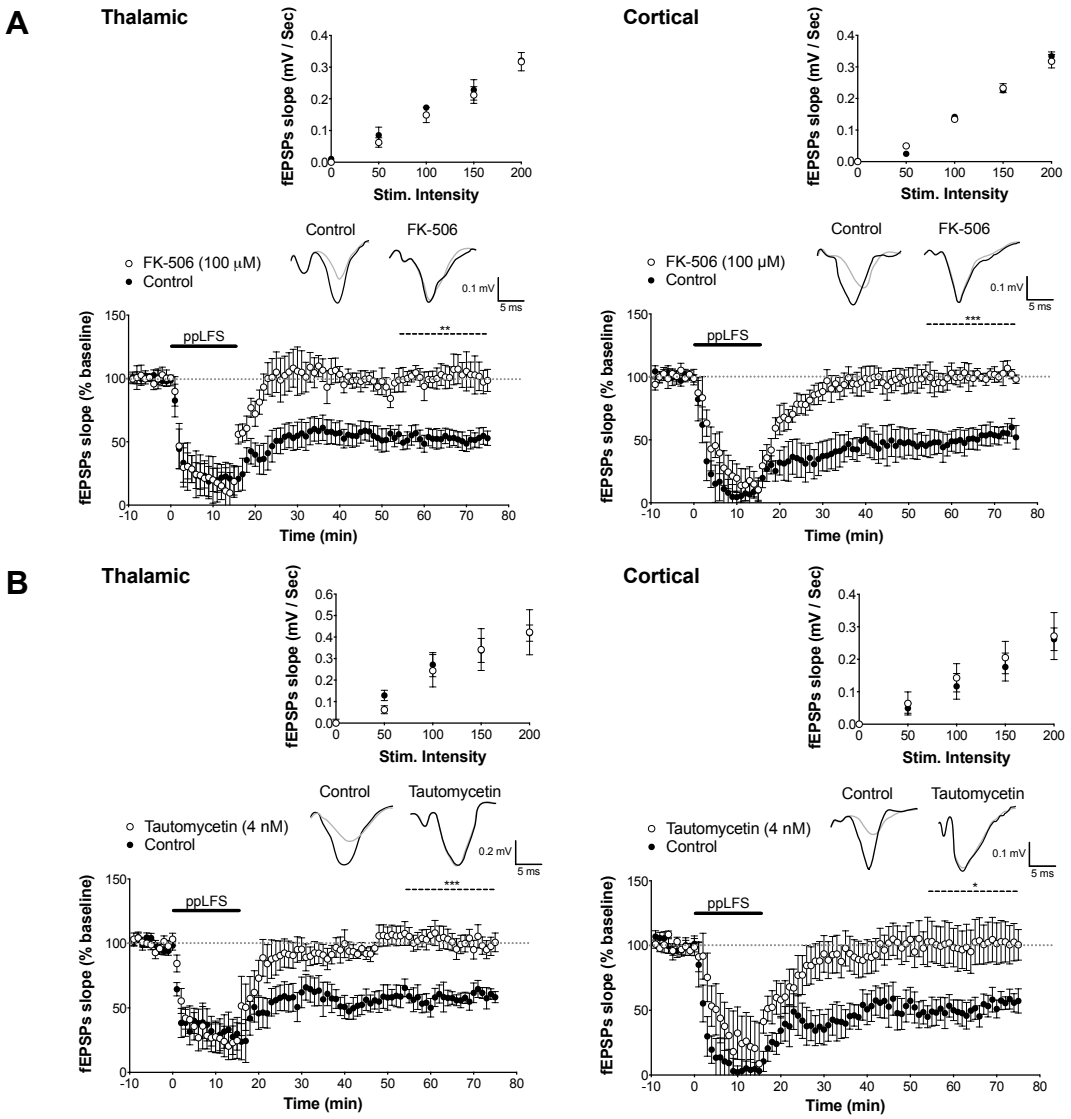

Figure 2. PP2B and PP1 involvement in LTD at the thalamic and cortical pathways in the lateral amygdala.

(A) The PP2B antagonist FK-506 (100 μM) blocks LTD induced at thalamic afferents (left panel, control: n = 5; FK-506: n = 5) and at cortical afferents (right panel, control: n = 5; FK-506: n = 5). (B) The PP1 antagonist tautomycetin (4nM) blocks LTD induced at thalamic afferents (control: n = 5; tautomycetin: n = 10), and at cortical afferents (control: n = 7; tautomycetin: n = 8). Insets show I/O curves on top and below representative traces of extracellular field potentials averaged across 10 mins before ppLFS (black line) and the last 10 minutes of recording after ppLFS (grey line). Data represent mean ± SEM. *** = $p < 0.001$, ** = $p < 0.001$, * = $p < 0.05$.

5.2.3 LA-LTD depends on activation of different NR2 subunits at thalamic and cortical afferents

We next investigated the NMDAR subunit composition implicated in LA-LTD at both inputs. While NR2A-containing receptors have previously been suggested to be involved in LTP in different brain structures, NR2B-containing receptors are thought to be involved in LTD [121,123,124,187,188], particularly in LA-LTD at the thalamic input [121,132]. At the cortical pathway, however, the NMDAR subunit composition is still unclear [127,128]. To test whether NR2B is required for LTD at both pathways, we used the selective NR2B antagonists ifenprodil (10 μM) and Co101244 (1 μM). While both antagonists fully blocked LTD at the thalamic pathway (control: 54.7 ± 5.7%, n = 5; ifenprodil: 118.0 ± 16.0%, n = 8; Co101244: 98.0 ± 10.5%, n = 5, $p < 0.05$ in both cases, Figure 3), they had no effect on LTD at the cortical pathway (control: 55.2 ± 5.9%, n = 5; ifenprodil: 48.2 ± 5.2%, n = 6; Co101244: 52.5 ± 12.2%, n = 5, $p > 0.8$, Figure 3). Ifenprodil and Co101244 did not affect I/O curves, suggesting no effect on basal synaptic transmission (Figure 3A, insets). These results demonstrate that LTD at the thalamic pathway is NR2B-dependent, while LTD at the cortical pathway is not.

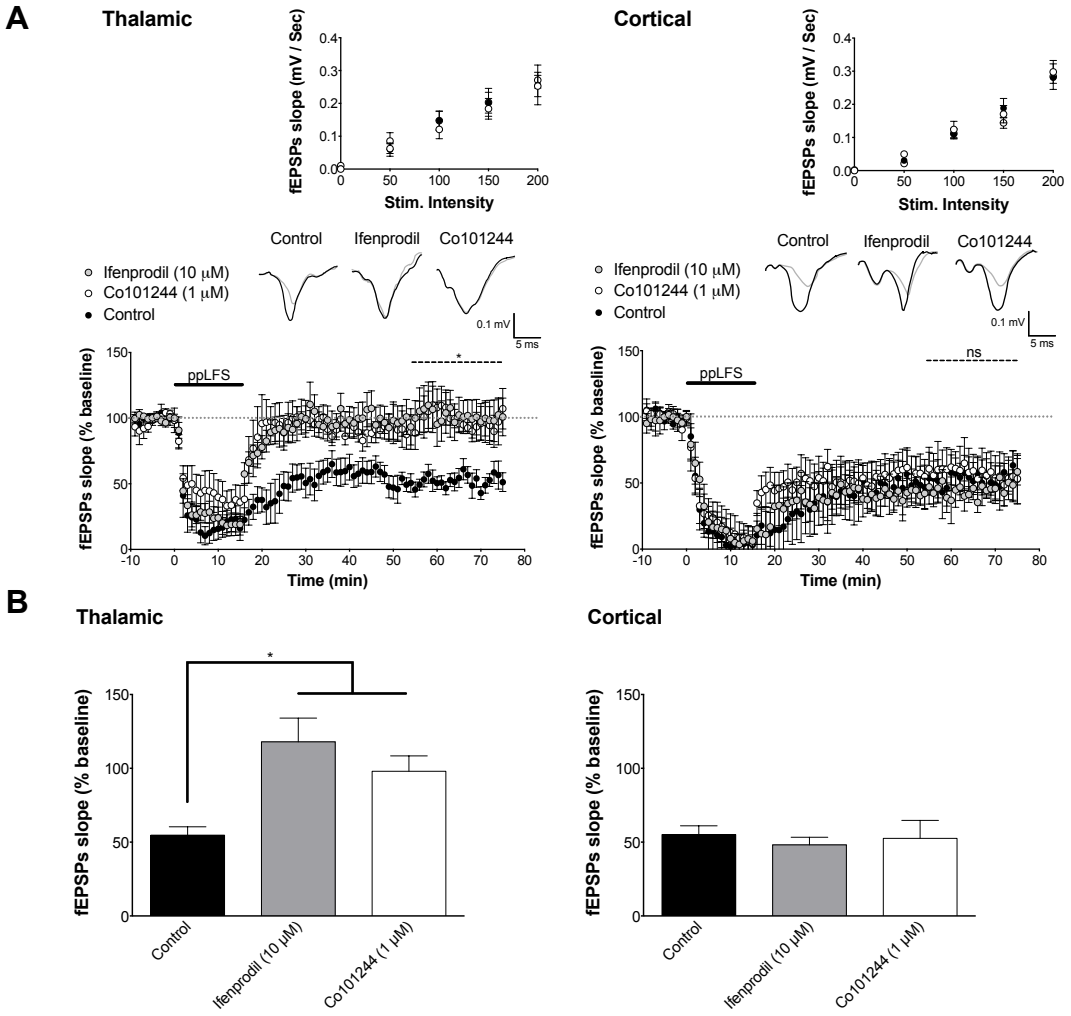

Figure 3. LA-LTD at thalamic inputs specifically depends on NR2B-containing NMDARs. (A) Ifenprodil (10 μM) and Co101244 (1 μM) block LTD at thalamic afferents (left panel, control: n = 5; ifenprodil: n = 8; Co101244: n = 5) but not at cortical afferents (right panel, control: n = 5; ifenprodil: n = 6; Co101244: n = 5). (B) Summary of the average fEPSP slope over the last 20 min of recording after ppLFS. Insets show I/O curves on top and below representative traces of extracellular field potentials averaged across 10 mins before ppLFS (black line) and the last 10 minutes of recording after ppLFS (grey line). Data represent mean ± SEM. * = $p < 0.05$, ns = non significant.

To determine which other NR2 subunits may be implicated in LTD at the cortical pathway, we next tested the contribution of NR2C/D subunits (NR2A was previously reported not to be involved in LA-LTD [121]). We used PPDA, a potent and dose-dependent selective NR2C/D antagonist [125,189]. We used PPDA at low concentration (0.25 μM) to preferentially antagonize NR2C/D subunits, and at high concentration (1 μM) to antagonize all NR2 subunits [189]. At 0.25 μM, PPDA fully blocked LA-LTD specifically at the cortical input, but had no effect at the thalamic pathway (Figure 4). In contrast, 1 μM of PPDA abolished LA-LTD at both pathways (thalamic, control: 45.2 ± 8.5%, n = 7; PPDA 0.25 μM: 44.3 ± 9.1%, n = 5; PPDA 1 μM: 113.6 ± 20.6%, n = 6, $p < 0.01$. Cortical, control: 41.6 ± 9.8%, n = 6; PPDA 0.25 μM: 113.3 ± 14.1%, n = 6, PPDA 1 μM: 107.1 ± 22.2%, n = 6, $p < 0.05$, Figure 4). I/O curves were not affected by PPDA at either concentration (Figure 4A, insets). Overall, these results indicate that LTD at the thalamic pathway depends on NR2B-containing NMDARs, whereas LTD at the cortical pathway depends on NR2C/D-containing NMDARs.

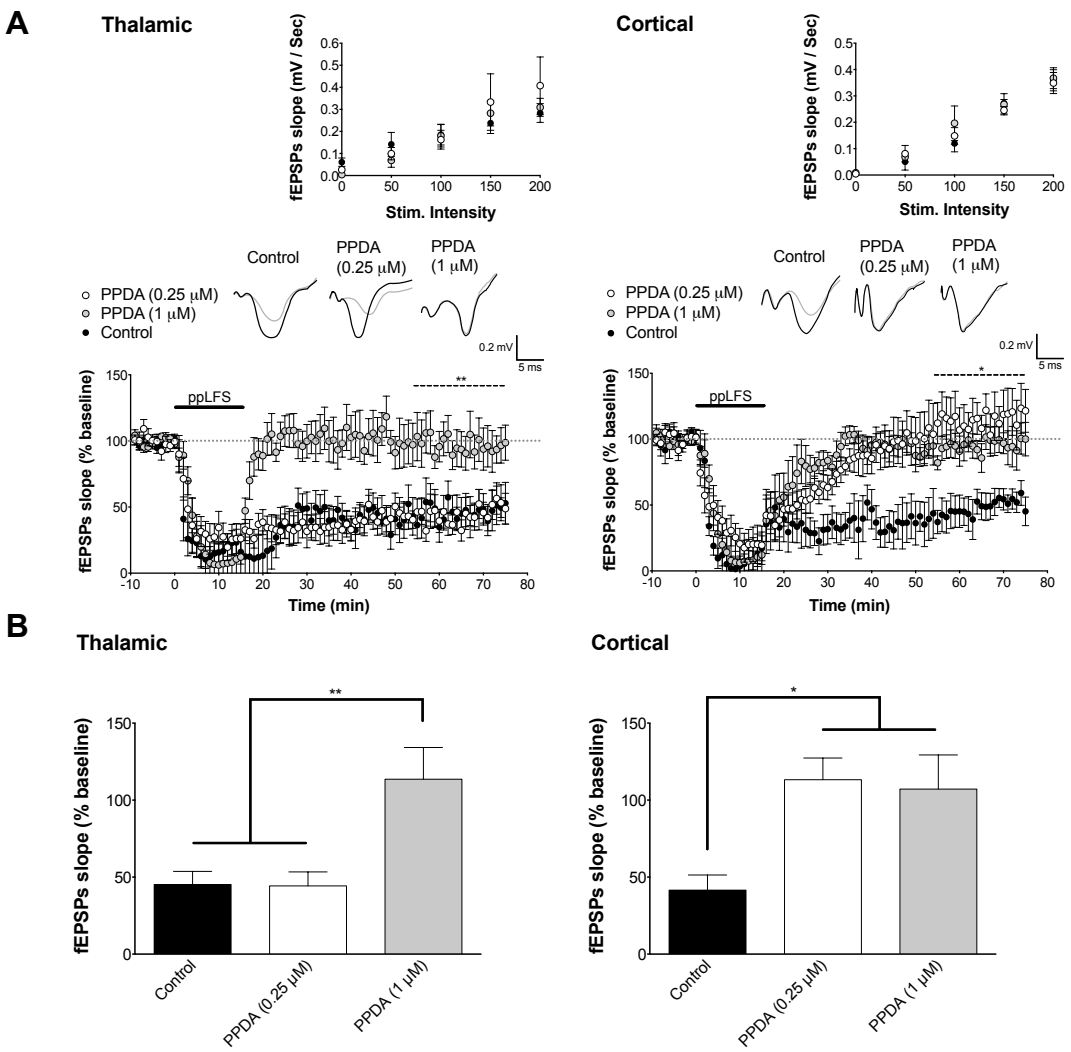

Figure 4. LA-LTD at thalamic inputs specifically depends on NR2C/D-containing NMDARs. (A) PPDA does not affect LTD at the thalamic pathway at the NR2C/D-selective low dose of 0.25 μM, but blocks LTD at the non-selective high dose of 1 μM (left panel, control: n = 7; PPDA low dose: n = 5; PPDA high dose: n = 6). PPDA fully blocks LTD and at cortical pathway at both doses (right panel, control: n = 6; PPDA low dose: n = 6; PPDA high dose: n = 6). (B) Summary of the average fEPSP slope over the last 20 min of recording after ppLFS. Insets show I/O curves on top and below representative traces of extracellular field potentials averaged across 10 mins before ppLFS (black line) and the last 10 minutes of recording after ppLFS (grey line). Data represent the mean ± SEM ** = $p < 0.01$, * = $p < 0.05$, ns = non significant.

5.2.4 Distinct loci of LTD induction at thalamic and cortical pathways

Although LTD is generally thought to be induced postsynaptically, it is known that NMDAR-dependent LTD can also occur presynaptically in several brain regions [133,134,190,191]. NR2B-containing NMDARs are mostly localized postsynaptically [130,132,192,193] and NR2C/D-containing NMDARs are mostly presynaptic [129,193,194] and have been implicated in presynaptic LTD in the somatosensory cortex [188]. Because NR2B and NR2C/D subunits are differentially involved in LTD at thalamic and cortical pathways, we hypothesized that LTD may have different loci of induction at thalamic and cortical pathways. We tested this hypothesis using whole-cell patch clamp recording in LA pyramidal neurons. The recorded cells (n = 32) showed a firing pattern and spike frequency adaptation characteristic of LA pyramidal neurons (Figure 5 A, [100,127]). The average resting potential of these neurons was -67.6 ± 4.3 mV. We observed a mono-exponential relationship between current transients and voltage steps, indicating that excitatory cells in LA behave as single electrical compartments (t1 = 40.65 ± 0.1 ms). Transients were also used to estimate series resistance (15.3 ± 4.23 MΩ), input resistance (235 ± 42.47 MΩ) and membrane capacitance (67.7 ± 16.8 pF), all typical values for LA excitatory cells [100,127].

Before assessing the locus of LTD induction, we examined whether LTD can be induced in individual excitatory LA neurons with the ppLFS protocol in current clamp configuration, and whether it depends on NMDARs. ppLFS induced a robust and persistent LTD in LA neurons, which was similar at thalamic and cortical inputs (thalamic: 47.38 ± 9.74%, n = 4; cortical: 56.2 ± 4.6% n = 5, $p > 0.3$, Figure 5B). LTD was blocked by D-APV, confirming that it

is NMDAR-dependent (thalamic: D-APV: 112.5 ± 3.0%, n = 3, $p < 0.001$; cortical: D-APV: 108.1 ± 6.2%, n = 3, $p < 0.01$, Figure 5B).

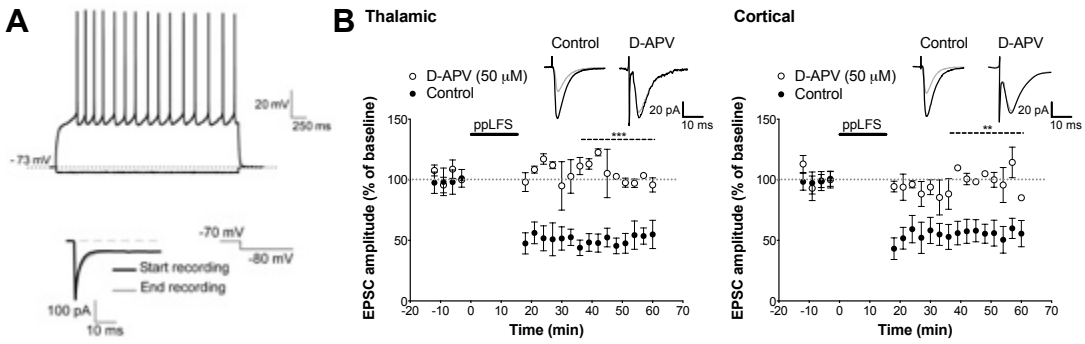

Figure 5. NMDAR-dependent LTD in single pyramidal cells.
(A) On top, response of a LA cell to current injection of -0.10 and +0.15 nA and below, hyperpolarizing voltage steps of 10 mV from a holding potential of -60 mV were used to measure series resistance. (B) D-APV (50 μM) blocks LA-LTD induced at the thalamic pathway (left panel, control: n = 4; D-APV: n = 3), and at the cortical pathway (right panel, control: n = 5; D-APV: n = 3). Insets show the average of 10 sweeps of a single cell recorded 10 min before (black) and 30 min after (gray) ppLFS.

Because postsynaptic plasticity depends on changes in postsynaptic intracellular calcium concentration, we examined whether LTD is postsynaptic by preventing calcium increase at the postsynaptic site using the membrane impermeable calcium chelator BAPTA (100 mM, dialyzed for 20 min before ppLFS). LTD at the thalamic pathway was fully blocked by BAPTA (control: 47.6 ± 6.7%, n = 4; BAPTA: 98.9 ± 5.7%, n = 4, $p < 0.01$) but it was not affected at the cortical pathway (control: 52.0 ± 6.6%, n = 5, BAPTA: 46.9 ± 6.1%, n = 5, $p > 0.5$, Figure 6A). These results suggest that the induction of LTD requires a postsynaptic rise in calcium at thalamo-LA synapses but not at cortico-LA synapses. To further assess the synaptic locus of LTD at thalamic

and cortical synapses, we selectively blocked postsynaptic NMDARs before LTD induction by intracellular dialysis of the activity-dependent NMDAR antagonist MK-801 (40 μM) into the pyramidal-like LA neuron. In the presence of MK-801, LTD was fully blocked at thalamo-LA synapses (control: 37.9 ± 13.5%, n = 3, MK-801: 104.3 ± 5.4%, n = 3, $p < 0.05$) but was not affected at cortico-LA synapses (control: 52.5 ± 14.2%, n = 3, MK-801: 54.8 ± 11.6%, n = 3, $p > 0.9$, Figure 6B), suggesting that LTD requires the activation of postsynaptic NMDARs at thalamic but not cortical synapses. Together, these results support a postsynaptic locus of LA-LTD at the thalamic pathway that likely depends on postsynaptic NMDARs, but a mechanism independent of postsynaptic NMDARs and independent of changes in postsynaptic calcium at the cortical pathway.

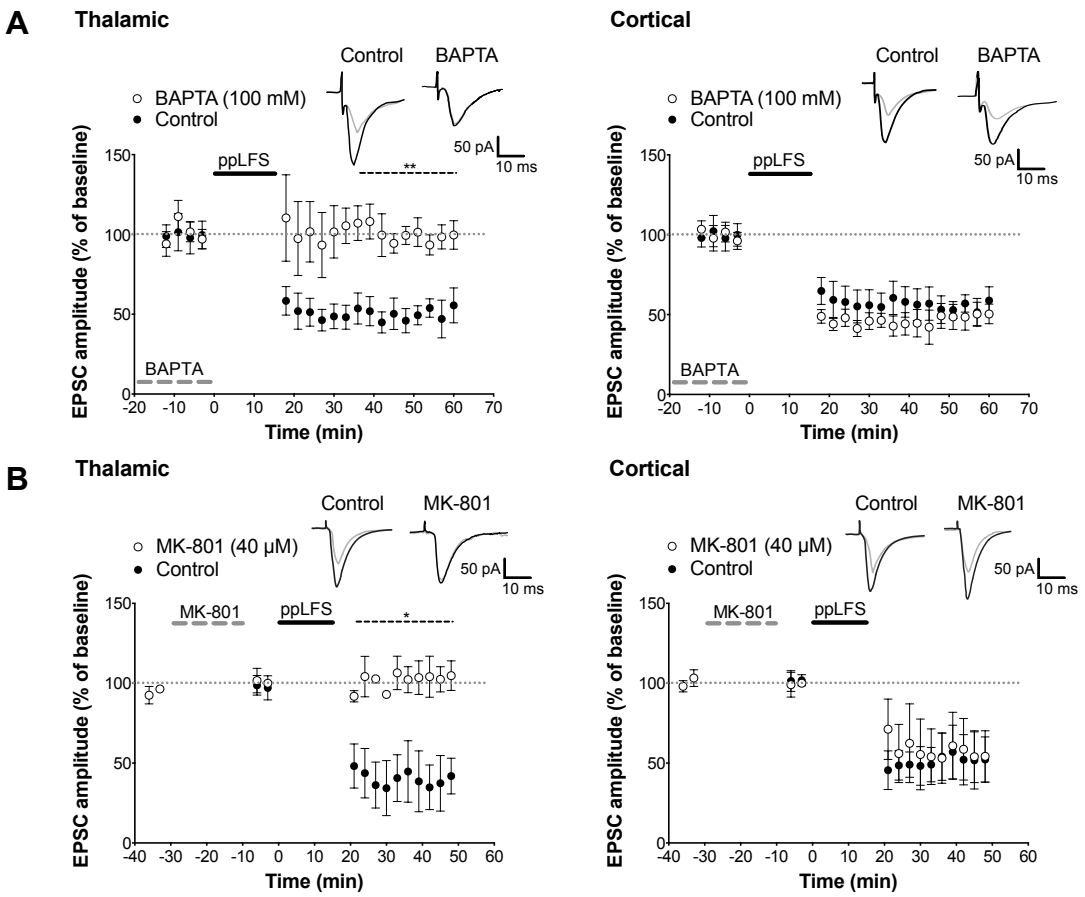

Figure 6. Distinct locus of LTD induction at thalamic and cortical pathways.

(A) Dialysis of BAPTA blocks LA-LTD induced at the thalamic pathway (left panel, control: n = 4; BAPTA: n =4), but not at the cortical pathway (right panel, control: n = 5; BAPTA: n = 5). Insets show averaged traces of 10 sweeps taken 10 min before (black) and 30 min after (gray) ppLFS. (B) Dialysis of MK-801 (40 μM) blocks LA-LTD induced at the thalamic pathway (left panel, control: n = 3; MK-801: n = 3), but not at the cortical pathway (right panel, control: n = 3; MK-801: n = 3). Insets show averaged traces of 10 sweeps taken 10 min before (black) and 30 min after (gray) ppLFS. Data represent mean ± SEM. *** = $p < 0.001$. ** = $p < 0.01$, * = $p < 0.05$.

5.3 Discussion

The protein phosphatases PP2B and PP1 are key players in the regulation of synaptic strength, and in the formation and the maintenance of memory traces [195-197]. Activation of PP2B/PP1 signaling is known to be necessary for LTD in different brain regions [109,155,157,198,199]. This study provides novel evidence that these phosphatases are also involved in the induction of LTD in LA at both thalamic and cortical pathways. This finding is in line with previous results showing that depotentiation at the cortical pathway in LA requires PP2B [108], and that PP2B and PP1 play an important role in memory tasks that depend on the amygdala including conditioned taste aversion and extinction of fear memory [109,140,200,201]. They also complement findings in the hippocampus that PP2B or PP1 inhibition enhances hippocampal LTP and memory performance in hippocampus-dependent tasks [146,185] but impairs LTD [160]. Taken together, these findings support the concept that PP2B/PP1 are key regulators of synaptic plasticity, and that their inhibition favors LTP and memory acquisition, but impairs LTD and memory extinction in both hippocampus and amygdala.

The pathways involving PP2B/PP1 in LTD are currently best understood in the hippocampus, in particular at Schaffer collaterals between CA3 and CA1 pyramidal neurons. NMDAR-dependent LTD in CA1 neurons results from a low increase in postsynaptic intracellular calcium concentration [84] leading to PP2B activation, followed by dephosphorylation of the PP1 inhibitor-1 and subsequent PP1 activation [83,143,156,157,202]. Once activated, PP1 dephosphorylates some of its targets in synaptic terminals [155], in particular, postsynaptic NMDAR and AMPAR subunits, leading to NMDAR

downregulation and AMPAR endocytosis, ultimately resulting in synaptic depression (for review, see [197]). Similar mechanisms may be engaged in the amygdala and would need to be investigated.

Our finding that LTD at the thalamic LA pathway is NR2B-dependent is consistent with previous studies [76,121,128]. NR2B is present in postsynaptic densities (PSD) in LA [130], and LTD at the thalamic pathway depends on NR2B-dependent postsynaptic AMPARs endocytosis [132]. Surprisingly, we observed that LTD induced at the cortical pathway is independent of NR2B signaling, since NR2B antagonists do not block LTD induction. Instead, we observed that blocking NR2C/D subunits fully prevents LTD at the cortical pathway, but does not affect LTD at the thalamic pathway. The observation that LTD at the cortical pathway is NR2B-independent contrasts with a previous report showing that antagonizing NR2B blocks LTD at both pathways in horizontal slices from adult mice [128]. This apparent discrepancy likely results from a different orientation of the slices leading to different sites of stimulation and recording. Specifically, placing the stimulating electrode laterally to the internal capsule in coronal slices primarily activates cortical afferents to LA, but in horizontal slices, it also activates afferents from the entorhinal and perirhinal cortex [128,203]. The spatial organization of excitatory and inhibitory connections within the LA depends as well on slice orientation [204,205]. It thus needs to be determined whether LTD differentially relies on NR2B or NR2C/D-containing receptors in the cortical pathway depending on the slice orientation. Given our clear finding that thalamic and cortical input to LA rely on different molecular and postsynaptic mechanisms, we postulate that projections to LA from the perirhinal and entorhinal cortex likely engage

different mechanisms as well. Notably, most electrophysiological studies in the amygdala are conducted in coronal sections rather than horizontal sections [73,95,107,121,184,206]. To our knowledge, this is the first report showing in coronal slices, a strong and reproducible induction of LTD at cortical afferents to LA by low-frequency stimulation, without the need of prior potentiation [73]. As highlighted by Müller et al (2009), this demonstrates that previous lack of LTD at cortical afferents [21,169] may be due to inadequate protocols for that specific pathway rather than an intrinsic failure to decrease synaptic transmission at cortical inputs to the LA. The availability of a robust LTD induction protocol at both input pathways to the amygdala in coronal slices shall allow further analyses of the mechanisms of LTD regulation in the amygdala.

Our observation that different NR2 subunits mediate the effects of ppLFS-induced LTD at both input pathways to the LA are in agreement with previous studies reporting differences in the molecular cascades at these pathways in LTP and depotentiation [73,77,184,207,208]. Although the distribution of NMDAR subunits in the amygdala remains largely unknown, the receptor kinetics at resting membrane potential is known to be different at cortical and thalamic pathways [127]. NMDARs at cortical inputs are less sensitive to magnesium blockade than at thalamic inputs, and the kinetic properties are akin to NR2C/D-containing NMDARs at the cortical pathway, but resemble NR2A/B-containing NMDARs at the thalamic pathway [115,127,209]. This is in agreement with our observation that NR2C/D-containing receptors seem to mediate LTD at the cortical pathway, but not at the thalamic pathway.

Presynaptic NR2C/D-containing NMDARs are believed to be involved in spike-timing dependent LTD in the cortex [188]. This prompted us to investigate the site of LTD induction at both LA pathways by whole cell patch-clamp recording. We observed that LTD induction occurs postsynaptically at the thalamic pathway, but is independent of postsynaptic calcium influx or postsynaptic NMDARs at the cortical pathway. These findings for LTD complement previous reports for LTP in the amygdala showing that LTP engages different pre- and postsynaptic mechanisms at thalamic and cortical pathways [77,88,184,206,210-213].

Although distinct NMDAR subunits and postsynaptic mechanisms are involved at thalamic and cortical afferents to the LA, both pathways converge onto a PP2B/PP1 signaling cascade. In the hippocampus, calcium influx through NMDARs, rather than other calcium channels, is specifically required for PP2B and PP1 activation [214]. It is possible that presynaptic calcium influx through NR2C/D-containing NMDARs, and postsynaptic calcium influx through NR2B-containing NMDARs, lead to the activation of PP2B/PP1 at the cortical and thalamic pathway, respectively, a possibility that will need to be tested in future experiments. Whether NR2C or NR2D subunits are localized presynaptically at cortical but not at thalamic afferents to LA will also need to be determined, as well as the molecular mechanisms downstream of PP2B/PP1 activation at both pathways. In the hippocampus, PP2B and PP1 have presynaptic and postsynaptic targets [195,215,216]. In hippocampal and cortical neurons, PP1 can dephosphorylate NR2B [131,138], resulting in a downregulation of NMDAR activity [138]. Similarly, in cerebellar granule cells, PP2B downregulates NR2C expression [217], thus it is possible that PP2B/PP1

dephosphorylate NR2B and NR2C subunits differentially in LA in response to LTD induction. Finally, the contribution of other receptors such as metabotropic glutamate receptors (mGluRs) in LA LTD cannot be excluded. Group I mGluRs have previously been shown to contribute to ppLFS-induced depotentiation at the thalamic pathway [74], while presynaptic group II mGluRs seem to be involved at cortical afferents [73]. Although mGluR-dependent LTD appears to involve tyrosine phosphatases rather than serine/threonine phosphatases such as PP2B and PP1 [84,175], they may also contribute to the differential molecular effects of ppLFS-induced LTD at both pathways.

LA-LTD is associated with the extinction of fear memory [73,74,76]. Since weakening and erasure of traumatic memory traces is critical for the management of anxiety disorders including PTSD [5,8,218-220], understanding the molecular mechanisms of LTD in the amygdala has important clinical implications. Our findings highlight the potential of therapeutically targeting PP2B/PP1 signaling to facilitate fear extinction learning in anxiety-related disorders [140,201,221].

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The authors declare no competing financial interests.

5.4 Material and Methods

Animals

For all experiments, adult male mice C57Bl/6 (8-12 weeks old) were used. Animals were housed in standard housing conditions in a temperature- and humidity-controlled facility on a 12h reversed light/dark cycle. Mice had free access to food and water. All procedures were carried out in accordance with the guidelines of the Veterinary Office of the Canton of Zurich, Switzerland, and approved by its Commission for Animal Research (License numbers 150/2006 and 105/2008).

Slices preparation

Mice were anesthetized with isoflurane 99.9% (AttaneTM) and rapidly decapitated. Immediately after decapitation, the brain was extracted and sectioned in coronal slices (400 µm thick for extracellular field recordings, 300µm for whole-cell patch clamp recordings) in ice-cold modified artificial cerebrospinal fluid (aCSF) containing 175 mM sucrose, 20 mM NaCl, 3.5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 1.3 mM MgCl2, and 11 mM D-(+)-glucose, and gassed with 95% O2/5% CO2 using a vibratome (VT 1000S; Leica Microsystems, Bannockburn, IL). Coronal slices were placed in a holding chamber at 34°C and incubated in normal aCSF containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM NaH2PO4, 26 mM NaHCO3, 1.3 mM MgCl2, 2.5 mM CaCl2, and 11 mM D-(+)-glucose, and continuously bubbled with 95% O2/5% CO2 at 34°C for at least 2.5 h, prior to recording. For recording, slices were transferred to a superfusion (1.5–2.5 ml/min flow rate) chamber (Warner Instruments) heated at 33.5-34°C and held below a platinum wire.

Electrophysiology

The recording electrode was placed in the dorsal part of the LA, and the stimulation electrodes were placed close to the internal capsule and externally to the capsule to stimulate fibers originating from the thalamus or auditory cortex, respectively (see Figure 1A). Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded from the dorsal part of the LA, while basal single-electrical stimuli at 0.05 Hz were applied at both pathways. After 10 min of stable baseline fEPSPs recording, paired pulse low-frequency stimulation (ppLFS, 900 pulses at 1Hz, interstimuli interval (ITI) of 40 msec) was used to induce LTD [128]. To test input specificity, ppLFS was induced at only one pathway (ppLFS pathway) whereas the other pathway was used as control and was stimulated with 0.05 Hz baseline stimulation. fEPSPs were recorded using a glass pipette (2-4 MΩ of resistance) filled with normal aCSF. An input/output (I/O) response curve was established by varying the intensity of single-pulse stimulation. The stimulus intensity that evoked a fEPSP equal to 50 % of the maximum response was used for all stimulations. fEPSPs were amplified (Multiclamp 700B), filtered (low-pass filter 1 kHz, high-pass filter 1 Hz) and digitized at 10 kHz (Axoclamp 10.2). Whole-cell recordings were performed in a blind approach [222]. The patch pipette (4-8 MΩ resistance) was filled with a solution containing (in mM): potassium gluconate 126, NaCl 4, MgSO4 1, BAPTA-free 0.1, BAPTA-Ca2+ 0.05, glucose 15, ATP 3, HEPES 5 (pH was adjusted to 7.2 with KOH) and GTP 0.1. Membrane potential was measured relative to an agar-bridge reference electrode. Reported membrane potential values were adjusted off-line for liquid-junction potentials (usually < 5 mV). Voltage-clamp mode was used to record evoked excitatory postsynaptic

currents (eEPSCs) from thalamic and cortical pathways. After stable baseline recording for at least 10 min, ppLFS stimulation was delivered in current-clamp configuration. Before and after ppLFS, series resistance was monitored by measuring the passive current transients induced by 10 mV hyperpolarizing voltage steps from a holding potential of -60 mV. Accepted deviations from this parameter in current transients recorded over the time-windows used for statistical analysis were less than 10% [223]. Data were recorded using an Axopatch 200B amplifier, sampled with a Digidata-1440 interface (sampling time = 250 msec for current-clamp recording, 10 msec for voltage-clamp recordings) and analyzed with P-CLAMP software (Axon Instruments, Foster City, CA) and Origin software (Microcal Software, Northhampton, MA).

Drug application

All drugs were bath applied at the indicated concentration starting at least 45 min before ppLFS and throughout recording, except D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV, 50 μM, Tocris), which was perfused for 10 min, starting 5 min prior to ppLFS delivery. The block specific NMDAR subunits, the NR2B antagonists ifenprodil hemitartrate (10 μM, Tocris) and Co101244 (1 μM, Tocris) were used, and the NR2C/D- antagonist [±]-cis-1-[phenanthren-2yl-carbonyl]piperazine-2,3-dicarboxylic acid (PPDA, Tocris, 0.25 μM to preferentially block NR2C/D-containing receptors and 1 μM to block NR2 subunits nonspecifically). FK-506 (100 μM, Tocris) and tautomycetin (4 nM, Tocris) were used to antagonize PP2B and PP1 activity, respectively [178,224]. The calcium chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA, 100 mM, Tocris) and the NMDAR open-

channel blocker MK-801 (Dizocilpine, 40 mM, Tocris) were dialysed in individual postsynaptic LA neurons for >10 min through the patch pipette. To specifically and fully block activated NMDARs during MK-801 dialysis, cells were progressively depolarized from the holding potential of -70 mV to +30 mV, while thalamic or cortical pathways were stimulated about 200-300 times to allow irreversible binding of MK-801 to activated postsynaptic NMDARs [184,225]. Consequently, the postsynaptic NMDAR component of EPSC activity was reduced after MK801 dialysis (charge transfer reduced by 28.6 ± 9.5% n = 3 for the thalamic pathway, and 17.2 ± 6.7% n = 3 for the cortical pathway). Cells were clamped again at -70 mV for another 10 min showing no significant change in the peak amplitude of AMPAR-mediated responses.

Data analysis

Data analysis was performed using Clampfit software (v10.2, Molecular Devices, Sunnyvale, DA), GraphPad Prism (GraphPad Software Inc., San Diego, CA), and Excel (Microsoft). For all recordings, fEPSP slope, and EPSP and EPSC amplitude were normalized to the average of baseline slope and amplitude, respectively. To improve the signal-to-noise ratio, data were averaged into 1 min bins. For each experiment, two to three slices per animal were recorded, one was always used as control slice and one or two slices received drug-treatment. For statistical analyses, individual animals (not slices) were considered biological replicates. For both extracellular field and whole-cell recordings, data are expressed as mean +/- SEM. Statistical comparisons were performed using Student's unpaired t-tests when two groups were compared. One-way ANOVAs were used when more than two

groups were compared. If significant, ANOVAs were followed using Duncan's post-hoc test. Significance was set to $p < 0.05$.

6 General discussion

6.1 In need of a novel therapeutic intervention for PTSD

Non-pharmacological treatment for PTSD and other anxiety disorders consists mostly of extinction-exposure therapy, which aims at facilitating fear extinction [14,226]. In this treatment, the patient is repeatedly presented with the CS in the absence of US or is required to talk about the general state of anxiety while simultaneously remembering the trauma or associated nightmares [14-16]. However, although this therapy can improve some symptoms of PTSD, in the majority of patients, extinction is not enhanced or patients relapse after their treatments with the mere passage of time (spontaneous recovery), changes of context (renewal) and the presentation of the US with which the CS has been initially paired (reinstatement) [15].

Distinct behavioral manipulations have been tested as well, such as performing fear extinction during reconsolidation of fear memory [87,219,227]. Reconsolidation is a process where other information or experience can be integrated to a memory trace, intended to modify retrieved memory by a process that integrates updated experience into long-term memory. Evidence suggests that this process destabilizes previously consolidated memory [87,218,219]. During reconsolidation, the synapses are thought to still be labile and therefore amenable to manipulation. By repeating fear extinction while the synapses are in this labile state, Monfils and colleagues showed a stronger and faster fear extinction than fear extinction alone (not performed during reconsolidation) [219]. This experimental approach

prevented the post-extinction return of fear. However, these results were not reproduced in several subsequent studies, performed in both rodents and humans [14,15]. The relapse effect endures even when the extinction-based therapy is combined with pharmacological interventions, such as antidepressants or NMDARs agonists (for example, d-cycloserine, DCS) [16].

The persistence of post-extinction relapse clearly suggests that extinction-exposure therapy is not sufficient to erase the originally acquired fear memory. Thus, novel interventions with the potential to erase the CS-US association acquired during fear memory formation are needed.

6.1.1 Is fear extinction a new inhibitory memory or fear erasure?

There is evidence that fear extinction either reverses conditioning-related changes (e.g., LTD) or/and induces plasticity at inhibitory synapses (e.g., LTP), so that to suppress conditioned fear responses. Since the extinction-based exposure therapies do not lead to a complete erasure of the previously acquired fear memory, it is thought that fear extinction mostly induces plasticity at inhibitory synapses, which involves NMDAR-dependent LTP as the main type of synaptic plasticity, and interferes with the existing conditioning fear memory to suppress conditioned fear responses [228,229]. However, they are conditions under which fear extinction results in a non-recoverable loss of the previously acquired fear memory; an underlying mechanism of fear extinction mediated by NMDAR-dependent LTD, which then leads to fear erasure [72-75].

It is possible that these two mechanisms underlying fear extinction coexist. For example, it could be that during fear extinction specific associations between the

CS and the US are inhibited, whereas others are erased. It could also be that fear inhibition and fear erasure occur at a distinct timing after conditioned fear memory. For example, it has been shown that extinction trials delivered shortly (e.g. 10min) after fear conditioning erase the previously acquired fear memory and prevent fear return. However, fear extinction induced 48 or 72 hours after fear conditioning does not prevent fear retrieval [230,231]. Although further studies need to clarify the differences or similarities between these two models of fear extinction, there is one shared feature that may be very interesting to investigate more in details: the molecular mechanisms in downstream of the NMDAR, since both models are mostly NMDAR-dependent, such as in LTP occurring in fear inhibition and in LTD in fear erasure.

6.1.2 PP1 as a potential therapeutic tool for PTSD

The major component of the molecular mechanisms underlying LTD is the PP2B/PP1 signaling cascade downstream of NMDAR activation [156,157]. By dephosphorylating many targets including AMPARs and NMDARs, PP1 is one of the main regulators of synaptic plasticity [155,165,166]. Its activation is required for LTD and strongly impairs LTP. As it is thought that LTD may be the underlying mechanism correlated with fear erasure and LTP the underlying mechanism related to fear inhibition, by increasing PP1 activity within the amygdala may facilitate fear erasure (since LTD would be improved) and accelerate fear inhibition (since LTP would be enhanced).

6.1.3 Future: towards fear erasure: LTP reversal by LTD induction

A causal link between synaptic plasticity and memory has recently been demonstrated [232]. In a fear conditioning paradigm, Nabavi and colleagues (2014) replaced a tone with an optogenetic delivery of either LTP or LTD at both thalamic and cortical afferents to the dLA. In their in vivo experiments, they emphasized the correlation between LTP and fear memory, and they showed that LTD induction is able to fully reverse, thereby inactivate the previously LTP-mediating fear memory. Both synaptic plasticity and the subsequent fear memory and extinction were abolished by systemic injection of a specific NMDAR antagonist, MK-801 [232]. This study confirms that elucidating the molecular mechanisms underlying LTD is of crucial importance for a future treatment for PTSD, aiming at erasing the previously acquired trauma-related fear memory.

To further investigate the role of PP1 in LTD in the dLA, a question remains to be clarified. Are the molecular mechanisms underlying LTP in fear memory reversed by LTD during fear extinction? To answer this question, see below a brief description of the molecular mechanisms underlying LTP at both thalamic and cortical inputs to the dLA, followed by hypothetical models of LTD at both afferents suggested by the findings of my doctorate thesis combined with data from literature.

6.1.3.1 LTP at the thalamic pathway to the dLA

At thalamo-dLA synapses, high-frequency stimulation (HFS) leads to an input-specific LTP, which is predominantly induced and expressed postsynaptically.

LTP induction requires postsynaptic depolarization, leading to Ca^{2+} influx into the postsynaptic compartment through the activation of NR2B-containing NMDARs, voltage-dependent Ca^{2+} channels (VDCCs) and from intracellular stores upon activation of group I metabotropic glutamate receptors (mGluRs). LTP expression is mostly triggered by new AMPARs exocytosis into the postsynaptic membrane [2,25,106,211]. Three major mutually interconnected signaling routes involve CaMKII, the protein kinase family of enzymes and tyrosine kinase (TK) pathways. PKA, PKC and PKMζ are responsible for AMPAR trafficking. The TK pathway leads to actin rearrangement under the control of Rho GTPases. The CaMKII signaling pathway leads to mitogen-activated protein kinase (MAPK) signal transduction pathways for gene expression and de novo protein synthesis via cAMP response element binding protein (CREB) activation [21].

6.1.3.2 LTP at the cortical pathway to the dLA

While the molecular mechanisms underlying LTP at thalamic inputs to the dLA have been well studied, at the cortical inputs they remain poorly understood. Depending on the protocols and the experimental procedures employed, LTP induction can require either a coincident pre- and post-synaptic activity, or a simultaneous activation of thalamic and cortical afferents [77,88,95,184,210]. The pairing-inducing LTP protocol, by which a presynaptic stimulation is associated with a postsynaptic depolarization, gives rise to a postsynaptic LTP induction in the LA that may be similar to the one at thalamo-LA synapses, where the activation of NMDARs and VDCCs are required. However, its expression implies

both pre- and postsynaptic mechanisms, such as an increase in glutamate release and the recruitment of new postsynaptic AMPARs [88,95,184,210].

However, when thalamic and cortical afferents are simultaneously stimulated, it results to an input-specific LTP that is exclusively induced and expressed presynaptically [184,213]. This form of LTP does not require postsynaptic depolarization, neither postsynaptic NMDARs activation, nor an increase in postsynaptic Ca^{2+} concentration. However, it needs presynaptic NMDARs activated by glutamate release from thalamo-dLA projections [184]. Thus, the associative coactivation of thalamic and cortical pathways leads to a heterosynaptic activation of presynaptic NMDARs at cortical inputs from the thalamic ones. Furthermore, it has been suggested that this type of LTP is modulated by presynaptic $GABA_B$Rs-mediated inhibition, which likely behave in parallel to presynaptic NMDARs, and probably intercede a direct inhibition of presynaptic Ca^{2+} channels, such as presynaptic VDCCs [213]. In addition, there is evidence that the expression of this type of LTP is mostly triggered by a persistent presynaptic increase in the probability of neurotransmitter release and requires cAMP/PKA downstream signaling pathway. This cAMP/PKA signaling pathway leads to protein RAS-related 3 (Rab3)-interaction molecules 1α (RIM1α) as well as presynaptic VDCC activation [206,233]. RIM1α is a protein that interacts with a large number of active-zone proteins, which are involved in the neurotransmitter release, such as RAS-related 3A (Rab3A), mammalian uncoordinated 13-1 (Munc13-1), synaptogamin 1 and presynaptic VDCCs [234,235].

On the other hand, Cho and colleagues (2012) recently showed that a continuous, but not simultaneous, paired stimulation of thalamic and cortical inputs to the dLA (interstimulus delay of 15-20 msec), gives rise to a homosynaptic and postsynaptic LTP, which involves inositol 1,4,5 triphosphate-sensitive Ca^{2+} release from internal stores and postsynaptic Ca^{2+} influx through Ca^{2+} permeable kainate receptors (KAR) [236].

6.1.4 Suggested models of LTD at auditory afferents to dLA

Theoretically, if LTD reverses LTP, it may be possible that it reverses the molecular mechanisms underlying LTP at both thalamic and cortical afferents. Thus, shared mechanisms may exist and opposite changes as well, such as the recruitment of PPs for LTD and PK for LTP.

In my thesis, I investigated the molecular mechanisms underlying LTD in the dLA. Below I present hypothetical models of LTD occurring at thalamic and cortical inputs based on our results combined with data from the literature.

6.1.4.1 NMDAR-dependent LTD signaling cascade at thalamo-dLA synapses

The ppLFS at thalamo-LA synapses exclusively induces a postsynaptic LTD, undergoing a progressive and low postsynaptic Ca^{2+} influx through postsynaptic NR2B-containing NMDARs. This triggers a CaM signaling pathway [107,216,237] leading to the activation of PP1 targeting complex, which likely contains A-kinase anchored protein (AKAP) as the PP2B and PP1 targeting protein and PKA as the protein kinase responsible for the I1 activation and GluR1 phosphorylation [238-241]. The activation of PP1 is under the control of PP2B, which dephosphorylates I1

and PKA, leading to their inhibition. In turn, PP1 dephosphorylates postsynaptic NR2B subunit at ser1303 and also GluR1 ser845 of the AMPARs. This leads to NMDARs down-regulation [138] responsible for LTD induction and AMPARs internalization [110,132] enabling LTD expression. The AMPAR endocytosis is probably performed by the recruitment of the clathrin-mediated endocytotic machinery, as seen in the hippocampus. In this brain region, AMPARs endocytosis is mediated by the dephosphorylation of phosphatidylinositol 4-phosphate 5-kinase c661 (PIP5Kg661; the major PI(4,5)P2-producing enzyme in the brain). The dephosphorylation is mediated by PP2B and PP1 located postsynaptically [214,215] (Figure A_1).

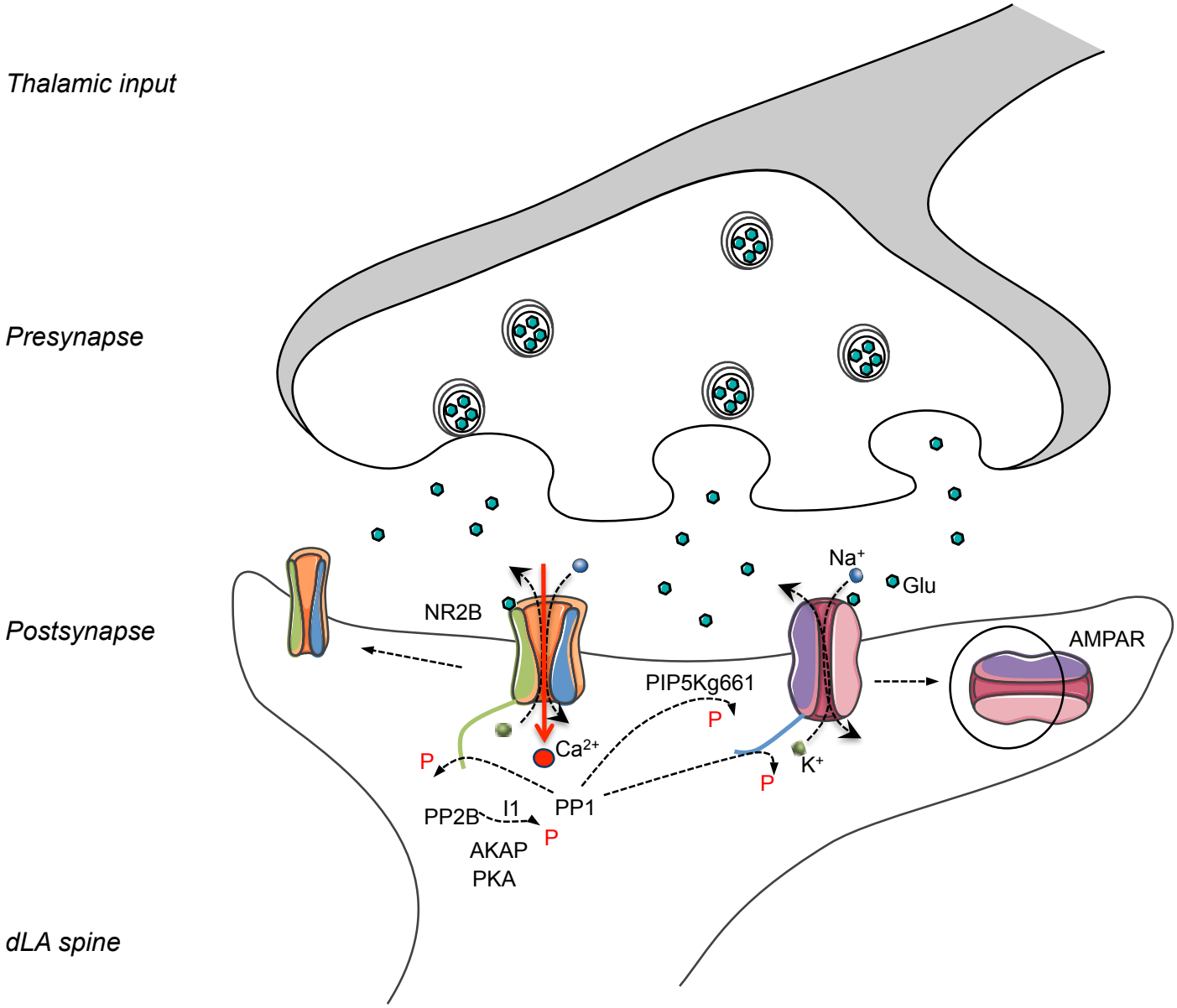
6.1.4.2 NMDAR-dependent LTD signaling cascade at cortico-dLA synapses

LTD at the cortical input is more complicated, since it involves presynaptic activity. The ppLFS at this pathway results in a presynaptic Ca^{2+} influx, likely through activation of presynaptic NR2C/D-containing NMDARs. As observed in the hippocampus, we hypothesize that the presynaptic increase in Ca^{2+} concentration may result in a rapid membrane depolarization, which will lead to the dephosphorylation of PIP5Kg661 [215]. At the presynapses, PIP5Kg661 is likely dephosphorylated by PP2B but not by PP1 [215]. This process enables a decrease in neurotransmitter release by the recruitment of the clathrin-mediated endocytotic machinery leading to the endocytosis of synaptic vesicles [215]. Furthermore, Fourcaudot and colleagues (2008) observed that both cAMP/PKA signaling pathway and RIM1α activation play a role in the regulation of

neurotransmitter release [233]. Since PP1 is not involved in the regulation of the synaptic vesicle endocytosis [215], its role may be principally postsynaptic.

In presynaptic LTD, a retrograde signaling mechanism may be required [84]. Indeed, a retrograde endocannabinoid (eCB) signaling involved in LTD has been observed in the LA [242-245], where the eCB released from the postsynaptic compartment binds to its receptor (CB1R) that is localized at the presynaptic side [133]. This binding may lead to presynaptic plasma membrane depolarization, leading to the activation of presynaptic NR2C/D-containing NMDARs, thereby activating a presynaptic Ca^{2+} signaling cascade. Thus, the role of PP1 at cortico-dLA synapses may be in controlling eCB release from the postsynaptic compartment [246], since the AMPARs internalization does not seem to be required at this synapse [73]. Nevertheless, we cannot exclude its role in the presynaptic compartment, maybe functioning in collaboration with PP2B, as PP2B may also be able to negatively regulate NR2C/D-containing NMDARs [217] (Figure A_2).

A₁


Thalamic input
Presynapse
Postsynapse
dLA spine
NR2B
Na+
Glu
AMPAR
PIP5Kg661
P
Ca2+
P
K+
P
PP2B
I1
PP1
P
AKAP
PKA

A$_2$

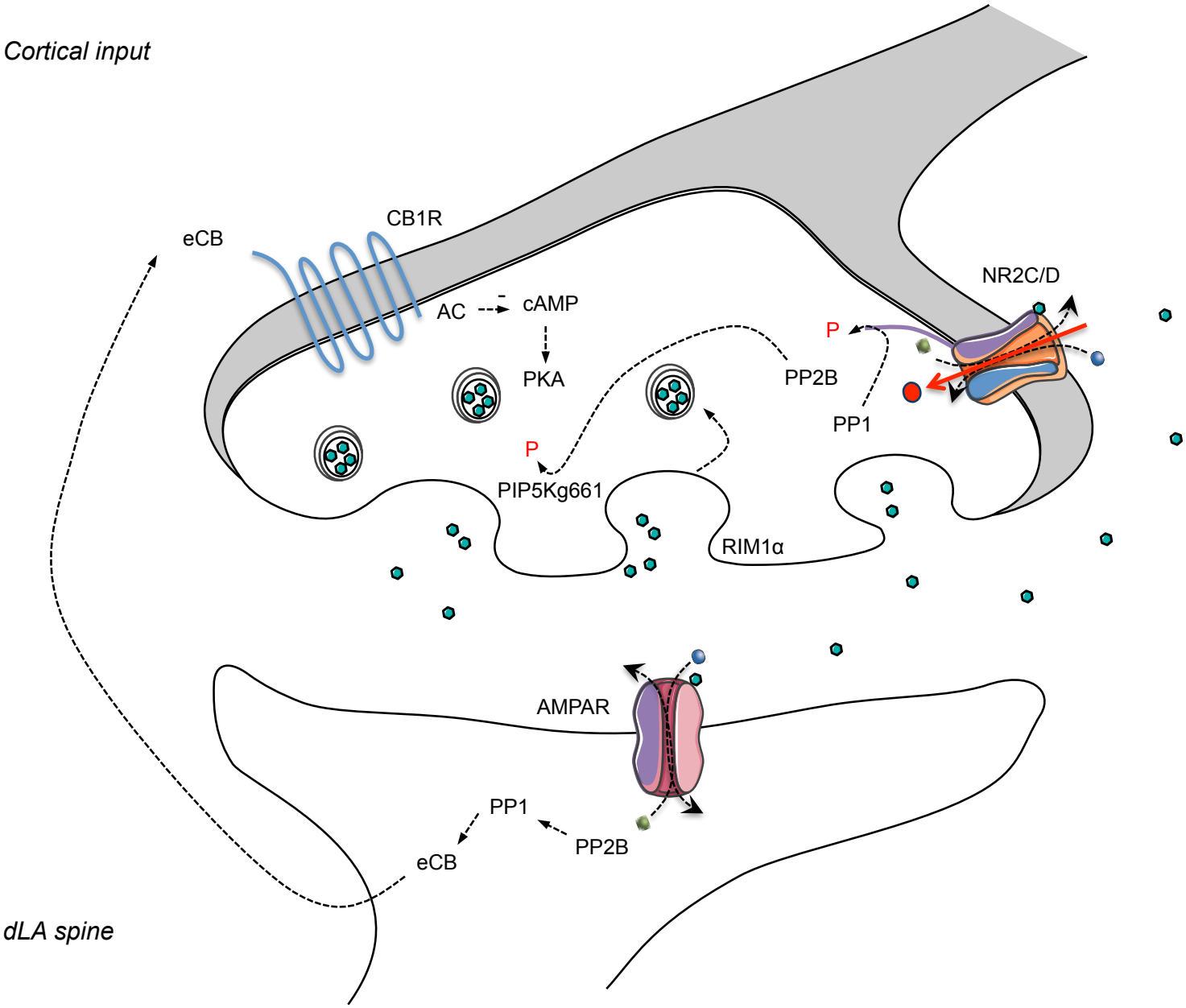


Figure A: see text

6.1.5 Towards fear erasure: understanding the molecular mechanisms of LTD in the dLA

Thalamic and cortical inputs converge onto one single principal neuron in the dLA, where they reach the same dendrite but distinct type of spines [77]. The comparison between LTP and our results related to LTD, combined with other literature lead to a striking observation: the thalamic and cortical pathways to the dLA share common features, which are potentiated during LTP and depotentiated during LTD. Especially at the thalamo-dLA synapses, where both LTP and LTD are input-specific and postsynaptic, and both depend on the activation of NR2B-containing NMDARs, and lead to opposite changes in AMPAR trafficking to the plasma membrane. At the cortical inputs to the dLA, the results obtained from LTP experiments depend on the protocol applied for induction. However, when simultaneous stimulation of both auditory inputs to the dLA is performed, the similarities with our findings are impressive. In this configuration, both LTP and LTD are purely presynaptic, and involve presynaptic NMDARs, which thanks to our results we identified as NR2C/D-containing NMDARs. Thus, the hypothetical model, which suggests LTD as the underlying mechanism of fear extinction, which reverses the previously acquired LTP-like fear memory is also observable in a molecular point of view. Therefore, for understanding more in depth the fear erasure model of fear extinction, a better understanding of the molecular mechanisms underlying LTD is needed. Some further experiments should 1) investigate more in details the role of PP1 in LTD at both pathways to the dLA, and 2) the role of PP1 inhibition or/and activation in fear-related behavior such as in fear extinction (see Outlook).

6.2 The potential role of PP1 in LTD in the dLA

Our findings suggest a crucial function for PP1 in amygdalar LTD induced at both thalamic and cortical afferents. It would be interesting to further investigate the exact actions of PP1 on AMPAR and NMDAR subunits during LTD in the dLA. However, the role of PP1 in LTD has been mainly explored in the hippocampus and mostly in relation to its ability to dephosphorylate AMPAR subunits, leading to receptor endocytosis [124,163,164]. Additionally, its role in NMDAR-specific regulation during hippocampal LTD is also being explored [138,165]. For a better overview of the potential role of PP1 in LTD in the dLA, I describe in the next chapters the well-established role of PP1 in hippocampal-LTD.

6.2.1 PP1-mediated hippocampal LTD regulation

The LTP/LTD-related phosphorylation and dephosphorylation events that regulate the trafficking and kinetic properties of the AMPARs and NMDARs (channel localization, conductance and opening probability) have been mostly studied in the CA1 hippocampal network [155,165,166,247]. The phosphorylation of ionotropic glutamate receptors is mediated mainly by the serine/threonine kinases (STKs) whereas PPs are responsible for dephosphorylation. Both kinases and phosphatases are activated upon Ca^{2+} influx into a cell. While STKs are active upon a high Ca^{2+} influx through NMDARs, the PPs cascade is activated after a low and progressive Ca^{2+} influx into the postsynaptic cell, as induced for example by LFS [156,157]. Once activated upon PP2B-mediated I1 dephosphorylation during LTD, PP1 [156,157] reverses the STKs-mediated

phosphorylation of AMPARs [247]. Whether PP1 interacts with the NMDAR subunits during LTD is still unknown.

6.2.1.1 Regulation of the AMPARs by PP1

In hippocampal LTD, PP1 specifically targets the GluR1 subunit of the AMPAR [162,163,247] on two phosphorylation sites, the ser831 and ser845 [247,248]. Ser831 is a target of CaMKII and protein kinase C (PKC), whereas ser845 is specifically phosphorylated by cAMP-dependent protein kinase A (PKA) [247,248].

PKA-mediated phosphorylation of ser845 has been shown to promote GluR1 cell-surface insertion and synaptic retention, increase channel open probability, and facilitate the induction of LTP [164,247,249,250], while dephosphorylation of ser845 is associated with AMPAR endocytosis and LTD [164,247,249,250]. CaMKII-mediated phosphorylation of ser831 increases channel conductance and regulates LTP [249,250]. The ser831 dephosphorylation may also lead to AMPAR endocytosis and LTD, although this remains a questionable topic [163,249,250]. Dephosphorylation of both ser831 and ser845 is mediated by PP1 [163].

Intriguingly, history of synaptic plasticity has a major impact on these specific PTMs occurring in LTP and LTD [247]. *De novo* induction of LTP increases phosphorylation of GluR1 ser831, although ser845 phosphorylation can also increase if LTP is induced following LTD induction [247]. Similarly, following *de novo* LTD, ser845 is specifically dephosphorylated by PP1, while specific PP1-mediated ser831 dephosphorylation occurs during depotentiation (LTD induction in previously potentiated synapses) [247]. In addition, both dephosphorylations in *de novo* LTD and depotentiation are abolished by D-APV perfusion [164]. As D-

APV fully abolishes hippocampal LTD [156,157], this implies that dephosphorylation of AMPAR is an NMDAR dependent mechanism for LTD [164,247,249].

6.2.1.1.1 Targeting AMPARs phosphorylation may prevent fear retrieval

Based on its importance in synaptic plasticity, the role of ser831 and ser845 phosphorylation in memory has been assessed in various paradigms, including water maze for evaluating spatial memory, and fear conditioning for associative fear memory [249,250]. Genetically modified mice with a double phosphomutation for ser831 and ser845 on the GluR1 subunit did not show any impairment in spatial learning compare to wild type littermates, but a defect in spatial memory retention after 8 to 24 hours after the behavioral paradigm [249]. This alteration in spatial memory retention was correlated with the instability of LTP in the same mutant mice compare to littermates [249]. Similar results were obtained in fear conditioning studies performed on mice injected with an antagonist peptide preventing GluR1 ser831 phosphorylation [250]. In these mice, by blocking GluR1 phosphorylation, the authors did not observe an impairment in fear memory and its extinction but an impaired fear renewal after fear extinction [250]. This is important, because fear renewal is one of the common relapse effects observed in patients treated with extinction-based exposure therapies [14,15]. Fear renewal is mainly due to a change of context from where the therapy has been conducted to a context reminiscent of the traumatic experience. Whether GluR1 subunit phosphorylation status affects other types of post-extinction relapse effects such as spontaneous recovery and reinstatement, remains to be elucidated.

Nonetheless, preventing GluR1 phosphorylation could present a means to avoiding fear memory retention, and may prevent relapse after extinction. This is also in line with our hypothesis regarding enhancement of endogenous LTD and fear memory extinction by increasing endogenous PP1 activity and subsequent AMPAR dephosphorylation.

6.2.1.2 Regulation of the NMDARs by PP1

The NMDAR phosphorylation/dephosphorylation events are required for NMDAR cell surface expression, trafficking, and stabilization at the synapse [106]. The serine/threonine phosphorylation sites on NMDAR subunits have been identified as substrates for many kinases such as PKA, PKC, PKB, CaMKII, cyclin-dependent kinase-5 (Cdk5), and casein kinase II (CKII). In contrast, the dephosphorylation is mediated by PPs [136]. The following section will present a brief overview of the PP1-mediated regulation of NMDAR subunits, focusing on NR2B and NR2C/D subunits, as we found them to be involved in amygdalar LTD. Importantly, no studies have yet reported a role of PP1 in dephosphorylating NMDARs during LTD. Most available studies have been performed *in vitro* without inducing any synaptic plasticity [131].

6.2.1.2.1 NR2B as a potential target of PP1 in LTD

CaMKII, PKC and CKII phosphorylate the NR2B subunit. The serine/threonine phosphorylation sites are ser1303, ser1323 and ser1480, where ser1303 is a target of CaMKII and PKC, ser1323 a target of PKC, and ser1480 a target of CKII. CaMKII and PKC phosphorylation potentiate NR2B-mediated NMDARs

currents [162], whereas phosphorylation on the ser1480 regulates NR2B-containing NMDARs surface expression by disrupting the interaction between NR2B and PSD-95 [251]. While the phosphorylation status has been extensively studied on the NR2B subunit, which PPs is responsible for the subunit dephosphorylation remains still poorly understood. New evidences on cell culture showed an interaction between PP1 and NR2B [131,138]. In these studies, PP1 was shown to be able to specifically dephosphorylate ser1303 leading to NR2B-containing NMDAR down-regulation, and a crucial role has been suggested for decreasing Ca^{2+} overload after ischemic conditions, thus conferring neuro-protection [131,138]. PP1 dephosphorylation is specific to ser1303, since it does not perturb the phosphorylation status on ser1323 and ser1480 [138].

These results are compatible with previous results related to hippocampal LTD, as NMDAR-dependent LTD in this brain area does not involve NMDAR internalization, but its downregulation [165]. Therefore, it may be interesting to further investigate the role of PP1 on NR2B ser1303 phosphorylation status during LTD at the thalamic pathway. It is possible that during LTD, activated PP1 dephosphorylates AMPAR leading to its endocytosis, and may also interact with ser1303 on NR2B to promote its downregulation (most likely by interrupting the interaction of the subunit with CaMKII [252]). Additionally, PP1 is also able to dephosphorylate CaMKII on its thr286, downscaling its activity [131,138].

6.2.1.2.2 NR2C/D as potential targets of PP1 in LTD

Little is still known about the regulation of the NR2C subunit by phosphorylation/dephosphorylation. However, recent studies identified two

phosphorylation sites at ser1244 and ser1096. PKC and PKA phosphorylate ser1244, whereas PKB acts on ser1096 [253]. The phosphorylation on ser1244 may be important in modulating channel properties of the NMDAR, since non-functional mutated ser1244 accelerates the kinetics of NMDA-evoked currents [253]. On the other hand, phosphorylation of ser1096 regulates NMDAR binding to 14-3-3ε [253]. 14-3-3ε belongs to a family of proteins able to mediate ER export of several proteins [126,253]. Indeed, S1096A mutation reduces the surface expression of the NR2C-containing NMDARs, suggesting that PKB-dependent phosphorylation site is responsible for the NMDARs trafficking to the plasma membrane by regulating the interaction between NR2C and 14-3-3ε [253]. Whether PP1 dephosphorylates NR2C at one of its serine residues remains unknown. No serine/threonine phosphorylation sites have yet been identified on the NR2D subunit [115,126], thus any involvement of PP1 in NR2C dephosphorylation remains highly speculative.

6.2.2 Discussion summary

In summary, upon NMDAR activation triggered by LTD induction, PP1 is activated and dephosphorylates many proteins involved in synaptic plasticity. Amongst them, the GluR1 subunit has been extensively studied and is crucial for synaptic plasticity and related pathologies. Beside the AMPARs, NMDARs are also regulated by PP1. Interestingly, whereas both PP2B and PP1 are responsible for AMPARs internalization, only PP1 plays a role in NMDAR-EPSCs mediated hippocampal LTD [165]. It may do so by interacting with NR2B ser1303 after LTD induction. However, this is still speculative. In addition, PP1 may also

interact with the NR2C subunit during LTD, since it has been shown that NR2C is involved in presynaptic LTD in the cortex [134,188] and in postsynaptic LTD in the hippocampus [125]. This possibility has not been explored yet.

D-cycloserine, an NMDAR agonist, has been used in pharmacotherapy associated with extinction-based exposure therapies in PTSD. Unfortunately, although this treatment brings improvements to the patient, it also shows strong side effects and does not prevent fear relapse [14,15]. This failure may be due to the fact that activating NMDARs may lead to distinct kinds of synaptic plasticity in different regions. However, a strategy aimed at regulating endogenous PP1 activity specifically in the amygdala may improve extinction, while avoiding post-exposure relapse. This will require further investigations to clarify the exact role of PP1 in LTD at both thalamic and cortical pathways (see Outlook).

I found that both PP2B and PP1 are necessary for LTD at both inputs to the dLA, although probably localized differently within the respective synapses. PP2B and PP1 may also have a distinct role between both inputs. Since distinct types of synaptic plasticity have been observed between thalamo- and cortico-dLA synapses, these findings combined with other studies in the field, may explain why thalamic and cortical afferents trigger distinct auditory or somatosensory information during fear conditioning. They may also explain, why there is not, up to date, an efficient therapy for PTSD, as two models of fear extinction exist and

because of the complexity of the mechanisms underlying synaptic plasticity at both inputs. As PP1 is activated at both pathways upon ppLFS, it is a promising future target in drug therapy for PTSD. Enhancing PP1 activity endogenously and specifically in the amygdala, as well as in the hippocampus and cortex, may strongly enhance fear extinction and avoid post-extinction relapse. The following chapter is designated to suggest some further experiments that could clarify the exact role of PP1 at both pathways during LTD.

7 Outlook

In summary, the present experiments have shown the need of PP1 activation for LTD at both inputs to the dLA. I also detected a different synaptic component of LTD at thalamic and cortical pathways, where LTD is postsynaptically and presynaptically induced at thalamo- and cortico- LA synapses, respectively. By blocking active NMDARs at the postsynaptic site, I further showed a distinct synaptic localization of the NMDARs at both inputs. Finally, by perfusion of different NR2 subunit antagonists, I observed a distinct NR2 subunit contribution to LTD; NR2B-containing NMDAR are localized at the thalamic pathway and NR2C- or NR2D- containing NMDARs at the cortical pathway. Taken together, the results of my thesis give rise, for the first time, to distinct molecular mechanisms of NMDAR-dependent LTD occurring at thalamic and cortical afferents to the LA, where PP1 is required for both.

7.1 Investigating the synaptic localization of NR2 subunits

Follow-up experiments will be necessary to detail the mechanisms of action of PP1 in regulating LTD after NMDAR activation. For doing so, I propose to first further investigate the synaptic localization of NR2B, NR2C/D and PP1 by immunohistochemistry and immunogold labeling electron microscopy [130,254]. The immunohistochemistry should be done on acute slices before and after the LTD induction protocol, by using subtype-specific NR2 subunits antibodies and a specific PP1 antibody. The immunogold labeling is hardly possible to perform on acute slices, due to methodological issues. However, using this technique, one

could identify the synaptic localization of NR2B, NR2C/D and PP1 on brains taken from mice previously subjected to fear extinction. This may confirm that LTD and fear extinction are strongly linked.

7.2 Assessing PP1-mediated dephosphorylation of AMPARs and NMDARs

Another interesting subject of investigation is whether PP1 dephosphorylates the GluR1 subunit of the AMPAR at thalamo-LA synapses, as demonstrated in the hippocampus [247], and / or if PP1 is able to dephosphorylate NR2B subunits at ser1303, or NR2C/D subunits during LTD. NR2B Ser1303 dephosphorylation mediated by PP1 has been shown in the hippocampus *in vitro* and in ischemic conditions, but has not been measured in response to LTD [138,255]. At cortical input to the LA, PP1 may interact with NR2C/D subunits. Whereas NR2B ser/thr phosphorylation sites have been extensively studied *in vitro* (and only ser1303 can be dephosphorylated by PP1 [138]), NR2C ser/thr phosphorylation sites have just recently been described in the cerebellar cortex [253] and existing ser or thr sites on NR2D subunits remain still unexplored [115,126]. On the NR2C subunit, there are two potential serine residues that may be dephosphorylated by PP1, ser1096 and ser1244. Dephosphorylation of these serine residues by PP1 may be associated with LTD at the cortical input to the LA, as dephosphorylation of one residue may lead to receptor endocytosis, and dephosphorylation of the other serine site may trigger its down-regulation [253]. PP1 dephosphorylation on NR2 subunits at either thalamic or cortical inputs can be assessed on protein

extracts from amygdala slices by western blot analysis using subunit/serine residues-specific antibodies. Slices can be collected before or after LTD induction, either in the presence or in absence of PP1 antagonists such as tautomycetin, or using slices from mice where PP1 is genetically inhibited [146].

7.3 Further evaluation of the loci of LTD induction

By blocking postsynaptic NMDAR using MK-801 and by chelating postsynaptic Ca^{2+} rise using BAPTA, we were able to identify that the cortico-dLA synapses do not require a postsynaptic rise of Ca^{2+} and do not activate postsynaptic NMDARs during LTD induction. These methods were similar to the ones used in a previous study, where the authors observed a presynaptic locus of LTP induction at cortical synapses onto principal dLA neurons [77]. However, further experiments are necessary to confirm our hypothesis, such as to measure mini excitatory postsynaptic currents (mEPSCs) and to perform paired-pulse facilitation, where its ratio is an indicator of presynaptic changes in the probability of neurotransmitter release. For example, in the case of thalamic-dLA inputs, our MK-801 and BAPTA manipulations abolished LTD, but it does not exclude the possibility of a parallel presynaptic mechanism. Furthermore, to confirm the localization of the presynaptic NMDARs at the cortical-dLA inputs (beside immunolabeling or immunofluorescence as cited above), delivering MK-801 at the presynapse with the same method we used in our experiments would be ideal, as performed in the neocortex [188] (see Main project). Finally, the retrograde messenger system should also be investigated related to our ppLFS-inducing LTD protocol, mostly at the cortical inputs to the dLA, by using specific

antagonists of CB1R. If antagonizing CB1R activity impairs LTD at the cortical input, it might mean that this receptor, localized presynaptically is needed for this type of LTD at the cortical input.

7.4 Studying the impact of PP1 in mGluR- and D1R-dependent LTD

As LTD, depotentiation and fear extinction require not only NMDARs but also mGluRs [73,74], it would be interesting to extend the research on the role of PP1 in the LA-LTD also to mGluRs. Pharmacological or genetic inhibition of PP1 may be used in mGluR-dependent LTD at both thalamic and cortical inputs. Group I mGluR involvement has been observed in LTD at thalamic inputs, whereas group II mGluR is required at the cortico-LA LTD [73,74]. The mGluR-dependent LTD can be induced pharmacologically by perfusion of group I or II agonists, such as 3,5-dihydroxyphenylglycine (DHPG) or 2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), respectively. If PP1 inhibition affects this type of LTD, it would show another role of PP1, which would we exclusively required in synaptic plasticity in the dLA, since in the hippocampus, PP1 is exclusively activated upon NMDARs activation. Moreover, PP1 inhibition in the hippocampus does not affect pharmacological or electrophysiological induction of group I, or group II mGluR-dependent LTD [155]. Interestingly, mGluRs affect dopaminergic neurotransmission [256]. Indeed, another important receptor to be taken into consideration is the dopamine receptor D1 (D1R), which has been shown to be involved in synaptic plasticity in the amygdala. At cortical pathway onto neurons from the BLA complex, the interplay between D1R and group II mGluRs can determine the direction of the synaptic plasticity (LTD or LTP). At a certain frequency the activation of presynaptic D1Rs

downregulates the presynaptic group II mGluRs [257]. Furthermore, Martina and Bergeron (2008) showed that D1R activation reduces NMDAR current amplitudes at cortical-dLA inputs [258]. Then, the same experiments implying PP1 inhibition in D1R-dependent LTD induced at either thalamic or cortical inputs may give an answer to whether PP1 is involved in this type of plasticity in the dLA. D1R activation can be induced by perfusion of dopamine or specific agonist such as SKF38393 [257,258]. There is evidence, in several brain regions (but not in the amygdala), that PP1 is regulated in dopaminergic synaptic plasticity [259]. In this case, PP1 regulation is mediated by the activation or deactivation of its specific inhibitor DARPP-32 (dopamine and adenosine 3′,5′-monophosphate-regulated phosphoprotein of 32 kDa). As in the hippocampus on inhibitor-1 (I-1, a specific endogenous inhibitor of PP1), DARPP-32 deactivation is mediated by PP2B [136,137]. Thus, if PP1 inhibition affects D1R-dependent LTD, it would give further details on the synaptic plasticity occurring at the cortical pathway to the dLA principal neurons, and it would give further insights on the regulation, either pre- or post- synaptic, of PP1 and PP2B.

7.5 Evaluating the role of PP1 in fear extinction

As PP1 appears to be a main regulator of NMDAR-dependent LTD in the amygdala, in vivo behavior experiments should aim at increasing PP1 activity in the LA to test whether fear extinction can be enhanced and fear relapse can be avoided. Fear erasure occurs mostly when fear extinction is applied shortly after fear memory acquisition [230,231]. A first assessment would be to quantify PP1 activity via PP1 activity assay [201] before and after fear extinction in wild-type mice

and this at different time points after fear memory acquisition (for example, immediately, 10 minutes, 30 minutes, 1 hour, 6 hours, 12 hours, 24 hours after fear conditioning). Secondly, the same experiments than above should be performed in mutant mice, in which PP1 is genetically overactivated, as performed in our lab but for PP2B [201]. At the end of these experiments, we would know 1) when PP1 activity reaches its highest degree during fear extinction, and 2) the effect of its overactivation on fear extinction time points. This may provide information about the potential of PP1 towards therapy. Nowadays, PP1 as a potential drug is wanted, as much as new strategies aiming at efficiently targeting PP1 and making it “drugable” are subject of new literature [260,261].

7.6 A possible role for epigenetic regulation of PP1

In addition to the acute effects of PP1 signaling, studying the epigenetic regulation of PP1 in amygdalar neurons during and following LTD may also lead to a better understanding of the molecular mechanisms of fear-related psychiatric diseases, such as PTSD. It is known that the Ca^{2+} entry through NMDARs results in the activation of specific signaling pathways leading to changes in gene expression (such as CREB), enabling long-term maintenance of memory. Those changes are mediated by epigenetic mechanisms, including histone acetylation, methylation and phosphorylation, and they are associated with transcriptional activation [141]. In the hippocampus, one of the key roles of PP1 in mediating epigenetic changes is its dephosphorylation of the histone 3 on the serine residue 10 at the CREB promoter site [142], enabling an increase of CREB expression, which regulates transcription of downstream genes involved in synaptic and structural plasticity. Similar findings were reported also in the lateral nucleus of the amygdala, where the specific inhibition of a nuclear pool of PP1 was shown to increase LTP when induced at the cortical input. This LTP enhancement was correlated with an increased phosphorylation of H3S10, with increased CREB expression and with enhanced memory on contextual and cued fear conditioning tasks [140]. These results suggest that PP1-dependent chromatin regulation may underlie disorders affecting emotional memory, by acting in several brain regions such as the hippocampus and the amygdala [197].

While epigenetic mechanisms have been implicated in numerous neurological and psychiatric disorders, such as Alzheimer's disease, schizophrenia and

depression [197], there is still no firm evidence for a direct involvement of epigenetic processes in PTSD. Nevertheless, Yehuda and Bierer have reported that the risk of having PTSD is associated with childhood adversity and PTSD in mothers [68,114], suggesting long-term, potentially heritable effects of childhood adversity on disease risk. Such long-term risk likely depends on epigenetic regulation, possibly also in germcells [262]. In our lab, we have developed a mouse model of early life traumatic stress to investigate the transmission of disease risk from parents to offspring. This model exposes mice to unpredictable maternal separation combined with maternal stress (MSUS) during the first two weeks of life. The effects of the early life traumatic experience are then assessed in adult mice that were directly exposed to maternal separation paradigm when they were pups (F1 generation), but also in their non-stressed offspring (F2 generation) generated by mating F1 males to unstressed naïve females. Across generations, MSUS mice display a wide array of behavioral disturbances, ranging from depressive-like behaviors to altered anxiety responses, impaired social interaction as well as impairments in cognitive function [263-266]. I have contributed to a recent project that demonstrated that MSUS mice display pronounced alterations in synaptic plasticity in the hippocampus and amygdala of both the directly exposed mice (F1) and their non-stressed F2 offspring ([267]; see Side project 2).

In agreement with the observed alterations in synaptic plasticity, gene expression and DNA methylation analyses showed marked differences between the offspring of MSUS and control fathers. These analyses identified two molecular

pathways involving calcium-mediated RAS activation and CaMKII-dependent CREB phosphorylation, which were suppressed in F2 MSUS offspring. It is possible that PP1 may be involved in the MSUS-induced plasticity effects. Pharmacological inhibition of PP1 during LTP experiments in MSUS and control mice could be used to test this possibility, which would represent the first evidence for a transgenerational role of PP1 regulation, with possible clinical implications for PTSD.

8 Other projects

This chapter contains two publications to which I contributed significantly.

8.1 Side project 1

Prion protein and Aβ – related synaptic toxicity impairment

Anna Maria Calella[1 ♯], Mélissa Farinelli[2 ♯], Mario Nuvolone[1 3 ♯], Osvaldo Mirante[2], Rita Moos[1], Jeppe Falsig[1], Isabelle M. Mansuy[2 *], Adriano Aguzzi[1 **]

(1) Institute of Neuropathology, University Hospital Zürich, Zürich, Switzerland.
(2) Medical Faculty of the University of Zürich, Department of Biology, Brain Research Institute, Swiss Federal Institute of Technology, Zürich, Switzerland.
(3) Department of Internal Medicine, Amyloid Center, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Pavia, Italy.

*Corresponding author: Tel: +41 44 635 33 60; Fax: +41 44 635 33 03
E-mail: mansuy@hifo.uzh.ch
**Corresponding author: Tel: +41-44-255 2107; Fax: +41-44 255 44 02
E-mail: adriano.aguzzi@usz.ch

♯ These authors contributed equally to this work.

Abbreviation title: Cellular prion protein and amyloid-β
Keywords: Alzheimer's disease; amyloid; prion protein; synaptic plasticity

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Attribution of data: I have performed all electrophysiological experiments in this study. I also contributed to the writing of the Materials and Methods.

8.1.1 Abstract

Alzheimer's disease (AD), the most common neurodegenerative disorder, goes along with extracellular amyloid-β (Aβ) deposits. The cognitive decline observed during AD progression correlates with damaged spines, dendrites and synapses in hippocampus and cortex. Numerous studies have shown that Aβ oligomers, both synthetic and derived from cultures and AD brains, potently impair synaptic structure and functions. The cellular prion protein (PrP^C) was proposed to mediate this effect. We report that ablation or overexpression of PrP^C had no effect on the impairment of hippocampal synaptic plasticity in a transgenic model of AD. These findings challenge the role of PrP^C as a mediator of Aβ toxicity.

Introduction

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder that culminates in cognitive decline with limited treatment options. Oligomeric amyloid-β (Aβ), derived from the b and g cleavage of b-amyloid precursor protein (APP), may drive AD pathogenesis by activating ill-defined signaling pathways (Walsh et al, 2005). Several molecules have been suggested to trigger the latter (De Felice et al, 2009; Shankar et al, 2007; Snyder et al, 2005). The cellular prion protein (PrPC) was reported to mediate the impairment of long-term potentiation (LTP) induced by synthetic Aβ oligomers in the hippocampal Schaffer collateral pathway (Lauren et al, 2009). Also, removal of PrPC from mice carrying APPswe and PSen1ΔE9 transgenes rescued early death and memory impairment (Gimbel et al, 2010).

PrPC is a membrane-anchored glycoprotein (Steele et al, 2007) crucial for axomyelinic integrity of peripheral nerves (Bremer et al, 2010). The remarkable finding that PrPC mediates Aβ-related synaptic toxicity was taken to suggest that interference with PrPC may represent a therapeutic option for AD (Lauren et al, 2009; Gimbel et al, 2010). However, upon intracerebral injection of synthetic Aβ oligomers, the absence of PrPC did not prevent deficits in hippocampal dependent behavioral tests (Balducci et al, 2010).

In view of these conflicting reports, we reasoned that a better understanding of the impact of PrPC onto AD may come from careful genetic analyses. Also, the utilization of a second, independent AD transgenic mouse model may help evaluating the universality of the observed phenomena. We therefore asked whether PrPC would modulate the degradation of LTP in an in vivo model of AD. We crossed mice lacking (Buëler et al, 1992) or overexpressing membrane-anchored (Fischer et al, 1996) or secreted PrP (Chesebro et al, 2005) with APPPS1+ mice coexpressing mutant APP (APPKM670/671NL) and mutant presenilin-1 (PS1L166P; Radde et al, 2006) which suffer from Aβ-dependent learning and memory deficits (Serneels et al, 2009; Table 1). We found that ablation or overexpression of PrPC had no effect on the impairment of hippocampal synaptic plasticity in a transgenic model of AD. These findings challenge the role of PrPC as a Aβ toxicity mediator.

8.1.2 Results and Discussion

LTP impairment and APP processing are not altered in absence of the cellular prion protein:

We crossed Prnpo/o mice lacking PrPC (Buëler et al, 1992) with APPPS1+ mice coexpressing mutant APP (APPKM670/671NL) and mutant presenilin-1 (PS1L166P; Radde et al, 2006).

Table 1. Genetically modified mice used in this study

Line	Description	Genetic modifications	Genetic background	References
APPPS1	Alzheimer's disease mouse model displaying Aβ42 cerebral amyloidosis	$APP^{KM670/671NL}$ transgene $PS1^{L166P}$ transgene (both on *Mmu2*)	C57BL/6	Radde et al (2006)
$Prnp^{o/o}$	Mouse lacking cellular prion protein	Introduction of a *neo* cassette replacing PrP codon 4–187 in the *Prnp* locus in *Mmu2* ($Prnp^{o}$ allele)	C57BL/6 and 129/Sv	Büeler et al (1992)
tga20	Mouse overexpressing cellular prion protein	Introduction of a *neo* cassette replacing PrP codon 4–187 in the *Prnp* locus in *Mmu2* ($Prnp^{o}$ allele) *Prnp* minigene on *Mmu17*	C57BL/6 and 129/Sv	Fischer et al (1996)
$tg44Prnp^{-/-}$	Mouse expressing GPI-anchorless prion protein	Introduction of a *neo* cassette into a *KpnI* site following residue 93 of PrP in the *Prnp* locus in *Mmu2* ($Prnp^{-}$ allele) Anchorless PrP transgene	C57BL/10 and 129/Ola	Chesebro et al (2005)

Mmu2 and *Mmu17*: *Mus musculus* chromosome 2 and 17, respectively; *neo*: neomycin phosphotransferase; $Prnp^{o}$ and $Prnp^{-}$ denote by convention the 'Zurich-I' and 'Edbg' knockout alleles of *Prnp*, respectively.

The resulting mice did not display any early death independently of the Prnp genotype (data not shown). High-frequency stimulation (HFS) of Schaffer collateral CA1 synapses induced an increase in field excitatory postsynaptic potentials (fEPSP) reflecting LTP in both 4-month-old Prnp+/+ and Prnpo/o mice (data not shown) as previously reported (Lledo et al, 1996). In contrast, age-matched APPPS1+Prnp+/+ (n = 6), APPPS1+Prnp+/o (n = 5) and APPPS1+Prnpo/o (n = 5) all exhibited defective LTP after HFS (114.23 ± 9.61; 111.72 ± 9.64 and 105.51 ± 12.23%, respectively; $p < 0.001$; Fig 1A). The fEPSP slopes during the first 2 min were similar in APPPS1+Prnp+/+ and wild-type mice (124.1 ± 7.0 and 184.8 ± 26.2%, respectively; $p > 0.05$), indicating that immediate post-tetanic potentiation was not affected.

Basal synaptic transmission as assessed by input–output curve analysis was normal in all mice (Fig 1B and C), confirming that the APPPS1 transgene induces a selective impairment in synaptic plasticity. In contrast to 4-month-old animals,

robust LTP was induced in 2-month-old APPPS1+Prnp+/+ (172.6 ± 14.6%; n = 5), APPPS1+Prnp+/o (168.9 ± 14%; n = 5) and APPPS1+Prnpo/o mice (204.4 ± 15.9%; n = 4) and was comparable to LTP in Prnp+/o (174.6 ± 7%; n = 5; Fig 1D). We conclude that the LTP impairment was age related, appeared only in mice carrying the APPPS1 transgene after >2 months, and was independent of Prnp gene dosage.

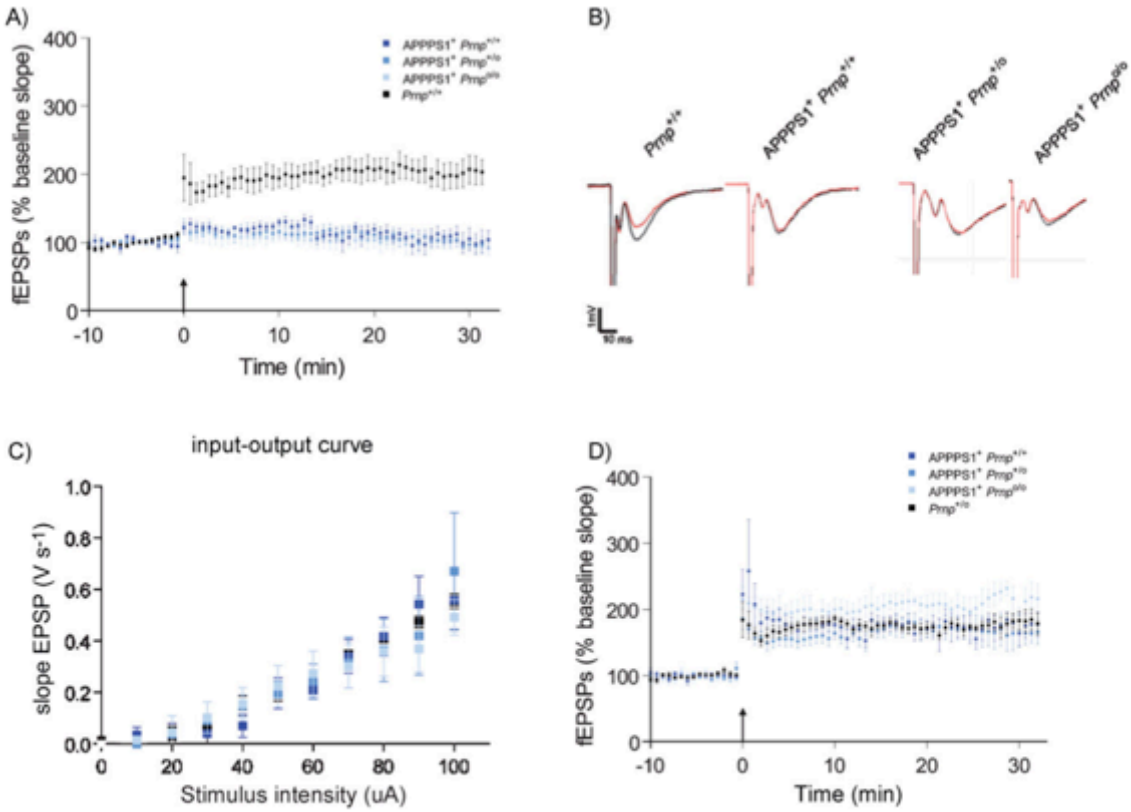


Figure 1. CA1 hippocampal LTP impairment in APPPS1^{+} mice occurs at 4 months of age and is not regulated by PrP^{C} expression.

A. CA1 hippocampal LTP was induced in acute slices from 4-month old *Prnp*$^{+/+}$ mice (black, n = 7), but was abolished in slices from age-matched APPPS1^{+}*Prnp*$^{+/+}$ (dark blue, n = 6), APPPS1^{+}*Prnp*$^{+/o}$ (blue, n = 5) and APPPS1^{+}*Prnp*$^{o/o}$ mice (light blue, n = 5). B. fEPSP traces before (red) and after (black) LTP induction. Calibration: 1 mV; 10 ms. C. Input–output curves

(stimulus intensity *vs.* fEPSP slope) indicative of normal basal synaptic transmission. D. Unaffected LTP in slices derived from 2-month-old APPPS1^{+}*Prnp*$^{+/+}$ (n = 5), APPPS1^{+}*Prnp*$^{+/o}$ (n = 5), APPPS1^{+}*Prnp*$^{o/o}$ (n = 4) and *Prnp*$^{+/o}$ mice (n = 5). These results indicate that LTP impairment in APPPS1+ mice was not a developmental defect, and occurred only after 2 months of age independently of *Prnp* gene dosage.

Many genetic polymorphisms affect APP processing and Aβ levels (Lehman et al, 2003). The APPKM670/671NL and PS1L166P transgenes map to mouse chromosome 2 (Mmu2; Radde et al, 2006) along with Prnp, and are linked to a quantitative trait locus that modifies Aβ levels (Ryman et al, 2008). Furthermore, PrPC itself was reported to directly interfere with APP catabolism (Parkin et al, 2007). Each of these factors, alone or in combination may modulate the production of soluble Aβ42, thereby indirectly affecting LTP impairment. However, we found that 2-month old gender-matched APPPS1+Prnp+/+ and APPPS1+Prnpo/o mice displayed similar levels of APP catabolites (Fig S1A) and soluble Aβ42 (Fig S1B). We conclude that the effects described here cannot be ascribed to any difference in APP generation or processing.

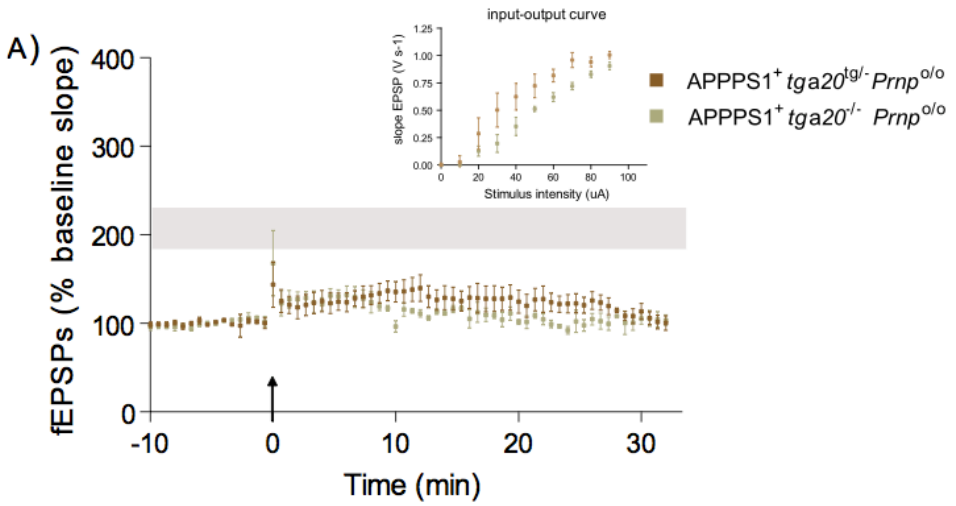
Evaluation of genetic confounders that might mask the impact of PrPC on LTP in 4-month-old APPPS1 mice

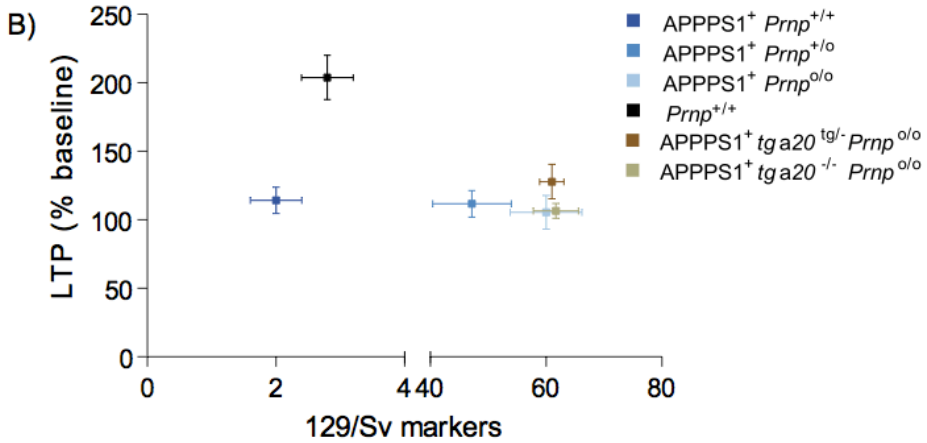
A genome-wide screen of 192 polymorphic microsatellites revealed that APPPS1+Prnpo/o mice contained significantly larger portions of 129/Sv-derived genome than APPPS1+Prnp+/+ mice (129/Sv-specific markers: average ± SEM: 60 ± 6.2 vs. 2 ± 0.4, respectively; $p < 0.001$). This genetic constellation may be taken to suggest that the above intercrosses have inadvertently introduced genetic biases affecting LTP independently of Aβ levels (Gerlai, 2002). However, in subsequent intercrosses, the content in genome-wide

129/Sv-specific markers was 55.3 ± 3.9 versus 41.7 ± 3.2 ($n = 7$ and 6, respectively; $p < 0.05$), yet this statistically significant difference disappeared upon exclusion of markers on Mmu2 (44.7 ± 3.8 vs. 38.0 ± 3.2, respectively; $p > 0.05$). This indicates that the latter mice, although not inbred, were genetically similar except for the Mmu2 genomic region that is closely linked to both Prnp and APPPS1 and does not desegregate easily from these loci by breeding. This genetic scenario may help explaining the differences in insoluble Aβ42 levels seen in F2 APPPS1+ mice with different Prnp genotypes generated by intercrosses of APPPS1+ and Prnpo/o mice (Fig S2; Ryman et al, 2008).

Transgenic PrPC overexpression disproves Mmu2 bias and does not aggravate APPPS1-induced LTP impairment

To formally discriminate between PrPC-dependent effect and potential confounders residing on Mmu2, we reintroduced PrPC into APPPS1+Prnpo/o mice via crosses to tga20 mice (Fischer et al, 1996) that carry a Prnp minigene on Mmu17 (Zabel et al, 2009) and overexpress PrPC about fourfold (Fig S3). LTP was again affected in 4-month-old APPPS1+tga20tg/−Prnpo/o (127.84 ± 12.61%; n = 4) and APPPS1+tga20-/-Prnpo/o littermates (106.56 ± 5.46%; n = 5; p = 0.137; Fig 2A). The genome-wide microsatellite patterns of these two groups of mice were indistinguishable even when Mmu2 markers were included (129/Sv-





specific markers: 61.0 ± 2.1 vs. 61.7 = 3.9, respectively; p > 0.05; Fig 2B),

indicating that any contribution by genetic confounders to the phenotype is unlikely.

Figure 2. LTP in 4-month-old APPPS1 mice expressing a PrP^{C} transgene.

A. At 4 months of age, LTP was impaired in slices from both APPPS1^{+}*tga20*$^{tg/-}$*Prnp*$^{o/o}$ (n = 4) and APPPS1^{+}*tga20*$^{-/-}$*Prnp*$^{o/o}$ (n = 5) but not in *Prnp*$^{+/+}$ slices (n = 7; LTP mean ± SEM from Fig 1A represented as grey ribbon). Basal synaptic transmission was normal as indicated by normal input– output curve (stimulus intensity vs. fEPSP slope). B. Average fEPSP slopes (percentage of baseline) at 10–25 min post-LTP plotted against the average number of 129/Sv specific markers for mice depicted in panel A and Fig 1A. In all investigated paradigms, LTP suppression by the APPPS1 transgene was independent of the genetic background.

To further explore the impact of supraphysiological levels on PrPC in LTP, we analyzed APPPS1+tga20tg/-Prnp+/o, which overexpress ca. sevenfold PrPC (Fig S3) and APPPS1+tga20-/-Prnp+/o littermates. These two groups of mice shared similar genomic microsatellite patterns (Fig 3A). At 4 months of age, LTP was significantly reduced in both APPPS1+tga20tg/-Prnp+/o and APPPS1+tga20-/-Prnp+/o littermates (149.41 ± 11.81%, n = 6 vs. 121.56 ± 11.65%, respectively; n = 4; Fig 3B). Expression of the tga20 allele showed a tendency towards improved LTP that was not statistically significant, without altering APP catabolites and soluble and insoluble Aβ42 (Fig 3C and D). Therefore, PrPC overexpression did not enhance Aβ-mediated LTP impairment; if anything, it may have marginally antagonized it.

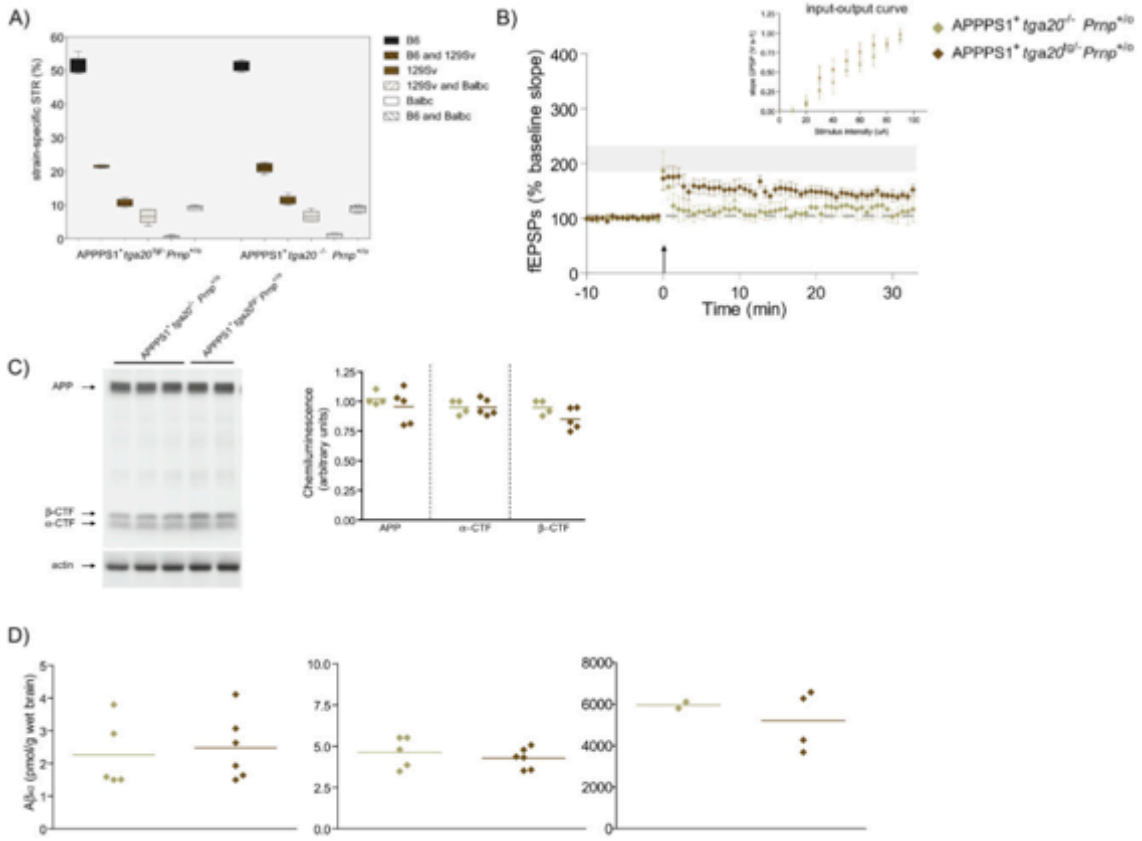


Figure 3. Analysis of 4-month-old APPPS1^{+} mice with supraphysiological levels of PrP^{C}.

A. Percentage of strain-specific microsatellites in APPPS1^{+}*tga20*$^{tg/-}$*Prnp*$^{+/o}$ (n = 6) and APPPS1^{+}*tga20*$^{-/-}$*Prnp*$^{+/o}$ (n = 4) mice is displayed by box plot. No significant difference in the genetic background of the two mouse strains was detected (Mann–Whitney U-test, two-tailed, $p > 0.05$). B. At 4 months of age, slices of both APPPS1^{+}*tga20*$^{tg/-}$*Prnp*$^{+/o}$ (n = 6) and APPPS1^{+}*tga20*$^{-/-}$*Prnp*$^{+/o}$ mice (n = 4) displayed reduced LTP when compared to *Prnp*$^{+/+}$ mice (n = 7); LTP mean ± SEM from Fig 1A represented as grey ribbon. Basal synaptic transmission was normal as indicated by normal input–output curve (stimulus intensity *vs.* fEPSP slope). All error bars: standard errors of the mean. C. APP expression and processing by secretases were similar in 4-month-old APPPS1^{+}*tga20*$^{tg/-}$*Prnp*$^{+/o}$ and APPPS1^{+}*tga20*$^{-/-}$*Prnp*$^{+/o}$ mice. Left panel: representative SDS–PAGE followed by immunoblotting using an APP C-terminal antibody detecting full-length APP and αβ-CTF; actin was used as loading control. Right panel: quantitation of chemiluminescence for APP, α-CTF and β-CTF. D. TRIS-soluble (left panel), detergent-soluble (middle panel) and insoluble (right panel) human $A\beta_{42}$ levels as assessed by ELISA. Each symbol denotes one individual mouse.

Overexpression of a secreted PrPC variant reduced the impairment of LTP in 4-

month-old APPPS1 mice

We next asked whether a soluble version of PrPC might intercept Aβ oligomers and interfere with synaptic toxicity. First we verified that interaction of PrPC with Aβ species (Balducci et al, 2010; Lauren et al, 2009) can occur in the absence of PrPC membrane anchoring. We therefore tested the binding properties of bacterially expressed recombinant full-length PrP (recPrP23–230). We found that recPrP23–230 bound low molecular weight Aβ42 species, and that binding was reduced by monoclonal anti-PrP antibodies (Polymenidou et al, 2008) raised against its N-proximal region (Fig S4). Also, we found that a shortened variant of recPrP lacking the amino-proximal residues 23–121 (recPrP121–230) did not bind Aβ42 (Fig S4). These results confirm that PrP, even when produced in bacteria and therefore, lacking all eukaryotic post-translational modifications including the addition of a glycolipid anchor, can efficiently bind Aβ species.

We then crossed APPPS1+Prnpo/o mice to mice expressing GPI-anchorless PrP (secPrP), which is secreted into body fluids of tg44Prnp-/- transgenic mice (Chesebro et al, 2005). The Prnpo and Prnp- alleles refer to the 'Zurich-I' (Buëler et al, 1992) and 'Edbg' (Manson et al, 1994) gene ablation events. We measured LTP in hippocampal slices derived from 4-month-old APPPS1+tg44tg/-Prnp-/o (n = 7) and APPPS1+tg44-/-Prnp-/o (n = 6) littermates with comparable genomic microsatellite patterns (Fig 4A). Remarkably, secPrP significantly suppressed the APPPS1-related LTP impairment (151.5 ± 11 and 108.5 ± 7.5%, respectively; $p < 0.05$, ANOVA and Tukey's multiple comparison test, see Fig 4B). The metabolism of APP and the levels of soluble and insoluble Aβ42 did not appear

to be altered by the tg44 transgene (Fig 4C and D), suggesting that secPrP exerted its beneficial effects interfering with the effectors of Aβ toxicity.

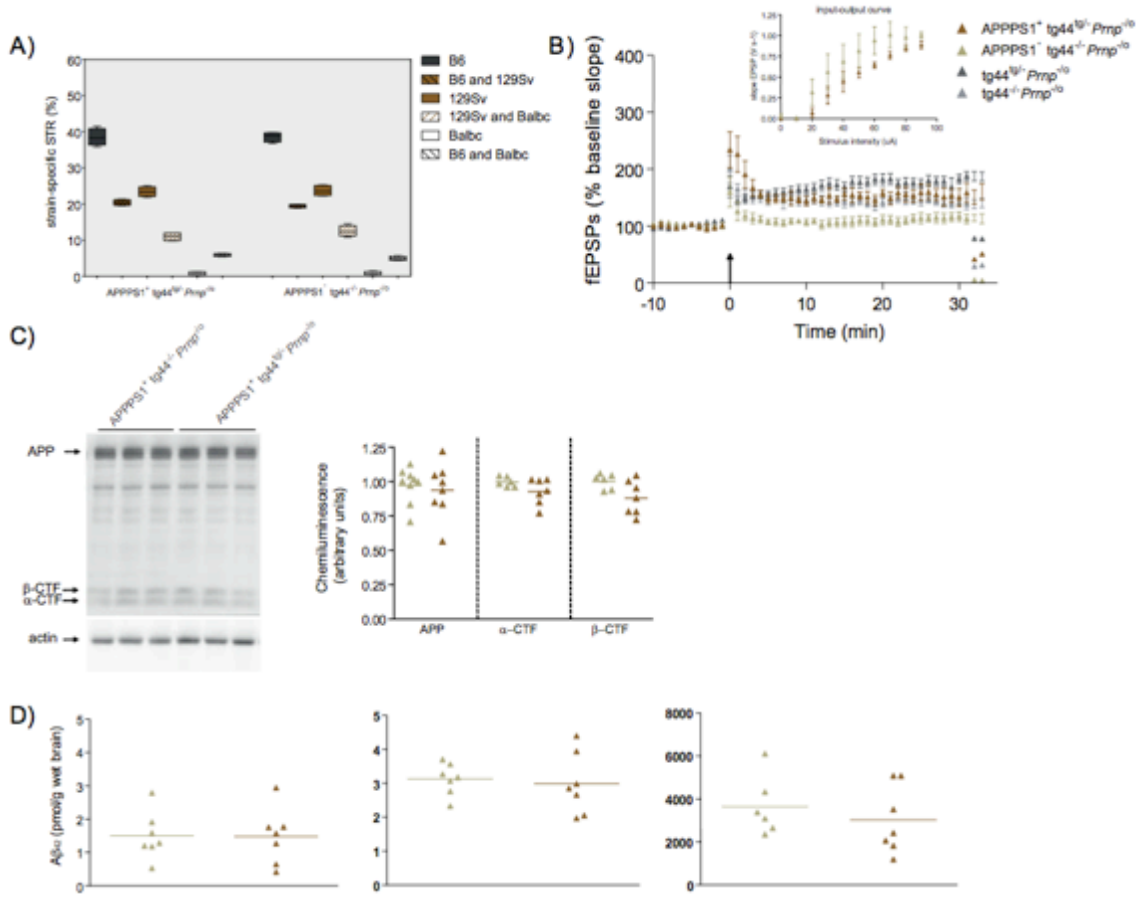


Figure 4. Anchorless soluble PrP^C reduces hippocampal LTP impairment in $APPPS1^+$ mice.

A. Percentage of strain-specific microsatellites in $APPPS1^+tg44^{tg/-}Prnp^{-/o}$ (n = 5) and $APPPS1^+tg44^{-/-}Prnp^{-/o}$ (n = 5) mice is displayed by box plot. No significant difference in the genetic background was detected (Mann–Whitney U-test, two-tailed, $p > 0.05$).

B. LTP was induced in slices prepared from 4-month-old $tg44^{tg/-}Prnp^{-/o}$ (n = 5) and $tg44^{-/-}Prnp^{-/o}$ (n = 7) mice, but was impaired in slices from $APPPS1^+tg44^{-/-}Prnp^{-/o}$ mice (n = 6) and partially rescued in $APPPS1^+tg44^{tg/-}Prnp^{-/o}$ (n = 7) mice. Basal synaptic transmission was normal as indicated by normal input–output curve (stimulus intensity *vs.* fEPSP slope). All mice were compound heterozygotes for the 'Zurich-I' ($Prnp^o$) and the 'Edbg' ($Prnp^-$) knockout alleles of *Prnp*.

C. APP expression and processing by secretases were similar in $APPPS1^+tg44^{tg/-}Prnp^{-/o}$ and $APPPS1^+tg44^{-/-}Prnp^{-/o}$ mice at 4 months of age. Left panel: representative SDS–PAGE followed by immunoblotting using an APP C-terminal antibody detecting full-length APP and C-terminal

fragments (αβ-CTF); actin was used as loading control. Right panel: quantitation of chemiluminescence revealed no difference in APP, α-CTF and β-CTF between the two groups. D. TRIS-soluble (left panel), detergent-soluble (middle panel) and insoluble (right panel) human $A\beta_{42}$ levels as assessed by ELISA. Each symbol denotes one individual mouse.

Despite decades of research, the cascade of events that originates with the aggregation of Aβ and leads up to cognitive impairment continues to be poorly understood. Many observations point to a crucial role of transmembrane signaling events triggered by aggregated Aβ. Several membrane proteins have been reported to bind soluble Aβ oligomers — thereby candidating as potential transducers of toxicity (Deane et al, 2004; De Felice et al, 2009; Shankar et al, 2007; Snyder et al, 2005; Yan et al, 1996). A great deal of excitement was generated by the recovery of PrPC from an expression screen for soluble Aβ oligomer binders, particularly as synthetic soluble Aβ oligomers were found to damage hippocampal LTP in a PrPC-dependent manner (Lauren et al, 2009) and impairment of spatial memory was rescued by genetic ablation of PrP in a mouse model of AD (Gimbel et al, 2010). However, the report that removal of PrPC did not prevent the behavioral deficits caused by intracerebral injection of synthetic Aβ oligomers (Balducci et al, 2010) challenged the role of PrPC as a crucial mediator of Aβ synaptotoxicity.

We crossed mice expressing human Aβ to mice lacking or overexpressing PrPC or a soluble variant thereof to evaluate if the impact of PrP is persistent also in another AD mouse model which suffer from Aβ-dependent learning and memory deficits (Serneels et al, 2009). The latter experimental paradigm may more closely approximate the human disease than the previously published models (Balducci et al, 2010; Lauren et al, 2009) as exposure to Aβ species is chronic

and uninterrupted over a protracted period, which is arguably more realistic than hyperacute exposure of brain tissue to Aβ. Furthermore, Aβ exists in AD brains as a vastly heterodisperse spectrum of assemblies ranging from monomers and dimers to oligomers and extremely large fibrillary aggregates, each one of which may partly contribute to the AD phenotype (Lesne et al, 2006; Shankar et al, 2008, 2009; Walsh et al, 2002). As the relative affinity of the various Aβ assemblies for PrPC is not known in detail, transgenic mice expressing many such assemblies may reveal phenomena that might go unrecognized in simpler systems, such as application of defined synthetic Aβ oligomers.

On the other hand, the genetic crosses described in our study and in previous work (Gimbel et al, 2010) may suffer from limitations. PrPC was reported to regulate β-secretase cleavage (Parkin et al, 2007), and overexpression may interfere with APP metabolism and Aβ levels, thereby indirectly affecting LTP impairment. Indeed, careful genetic quality control revealed a mouse-strain dependent effect on insoluble Aβ42 levels — a phenomenon that should be taken into account while interpreting results from mouse AD models. However, all mice analyzed in this study displayed similar levels of APP catabolites and Aβ42 independently of Prnp gene dosage. We also considered the possibility that potential confounders residing on Mmu2 might have introduced alterations of the experimental evaluation (Steele et al, 2007), a problem, which remains unsolved in the study by Gimbel et al. However, in our paradigm, genome-wide microsatellite analyses and expression of PrPC from the tga20 minigene on chromosome Mmu17 disproved any Mmu2 bias.

Additionally, one might argue that the exceedingly rapid amyloid pathology of APPPS1 mice used in our study leads to irreversible synaptic damage that is independent of Aβ oligomers and, consequently, of PrPC. However, the original report (Radde et al, 2006) and our observations indicate that immunohistochemically and biophysically recognizable amyloid deposition does not occur in APPPS1 hippocampi before 4–5 months of age (Fig S5). Therefore, at the time of our analysis, there was no massive amyloid deposition in the hippocampus. Furthermore, the rescue of LTP impairment by secPrP negates the possibility that an overly aggressive amyloid pathology precludes the evaluation of the role of PrPC in these mice.

The combined weight of all these results favours the conclusion that, however enticing, the hypothesis of PrPC being a crucial mediator of Aβ synaptotoxicity might be not universal.

8.1.3 Supplementary information

Supplementary Figure:

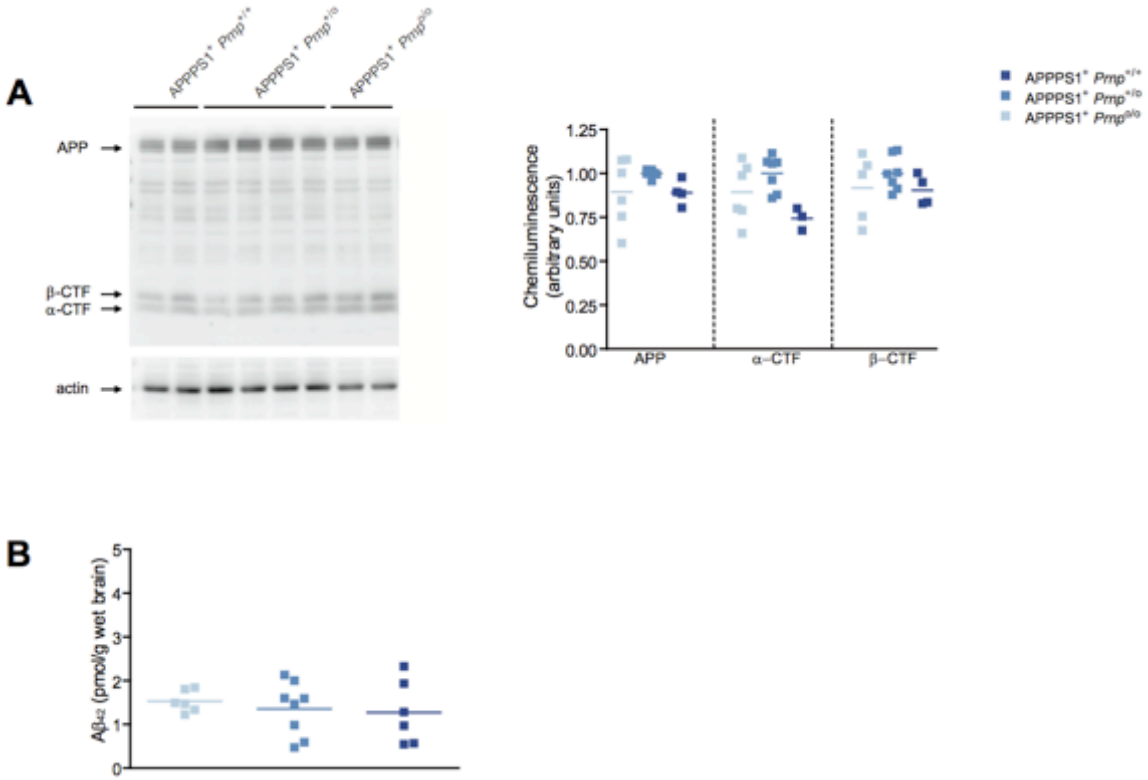


Figure S1. APP expression and processing in APPPS1+ mice is not affected by *Prnp* genotypes.

APP expression and processing by secretases were similar in 2-month-old APPPS1+*Prnp*+/+, APPPS1+*Prnp*+/o and APPPS1+*Prnp*o/o mice. (*A*) Full-length APP and C- terminal fragments (α-β CTF) are not affected by *Prnp* genotype. Left panel: representative SDS-PAGE followed by immunoblotting using an APP C-terminal antibody detecting full- length APP and α-β CTF; actin was used as loading control. Right panel: quantitation of chemiluminescence for APP, α-CTF and β-CTF. (*B*) Human soluble Aβ42 levels as assessed by ELISA. Each symbol indicates a mouse.

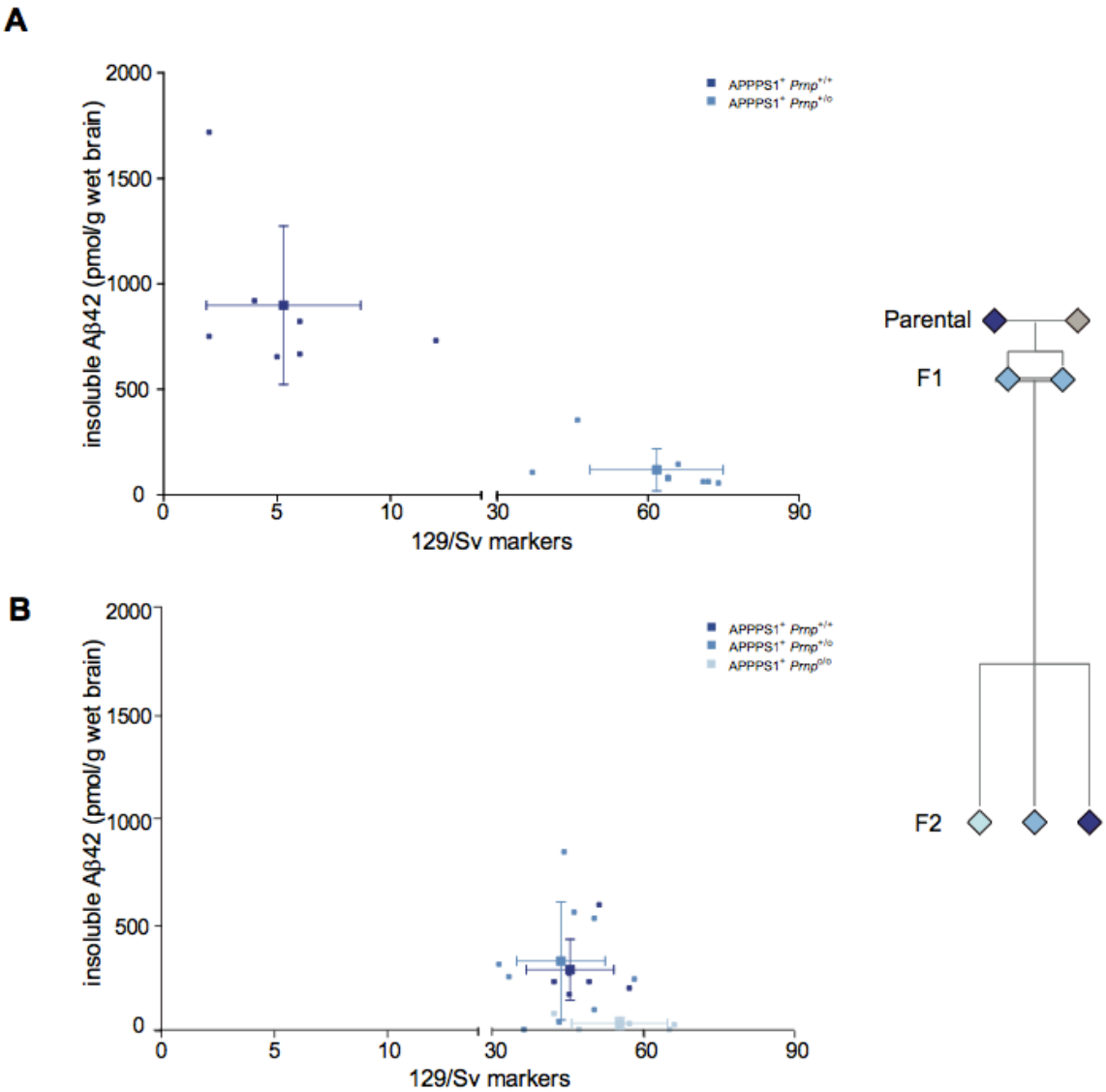


Figure S2. Genetic background associates with differences in insoluble Aβ42 levels in APPPS1+ mice.

APPPS1+ mice (on a C57B/6 background) were crossed with Prnpo/o mice (on a mixed C57BL/6 and 129/Sv background) to generate F1 and F2 mice as depicted in the pedigree. Insoluble Aβ42 levels are plotted against the number of 129/Sv specific microsatellite markers. Each symbol denotes a mouse. Average ± standard deviation for each group is displayed as well.

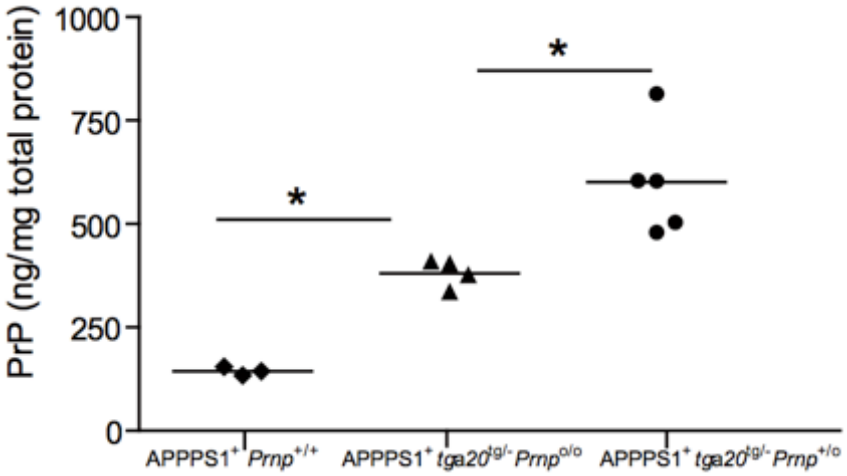


Figure S3. Overexpression of PrP in APPPS1+ mice.

Expression of PrPC in brains from APPPS1+Prnp+/+, APPPS1+tga20tg/-Prnpo/o and APPPS1+tga20-/-Prnp+/o mice were analyzed by ELISA. Each symbol indicates a mouse. Significance was determined by one-way ANOVA *p< 0.05.

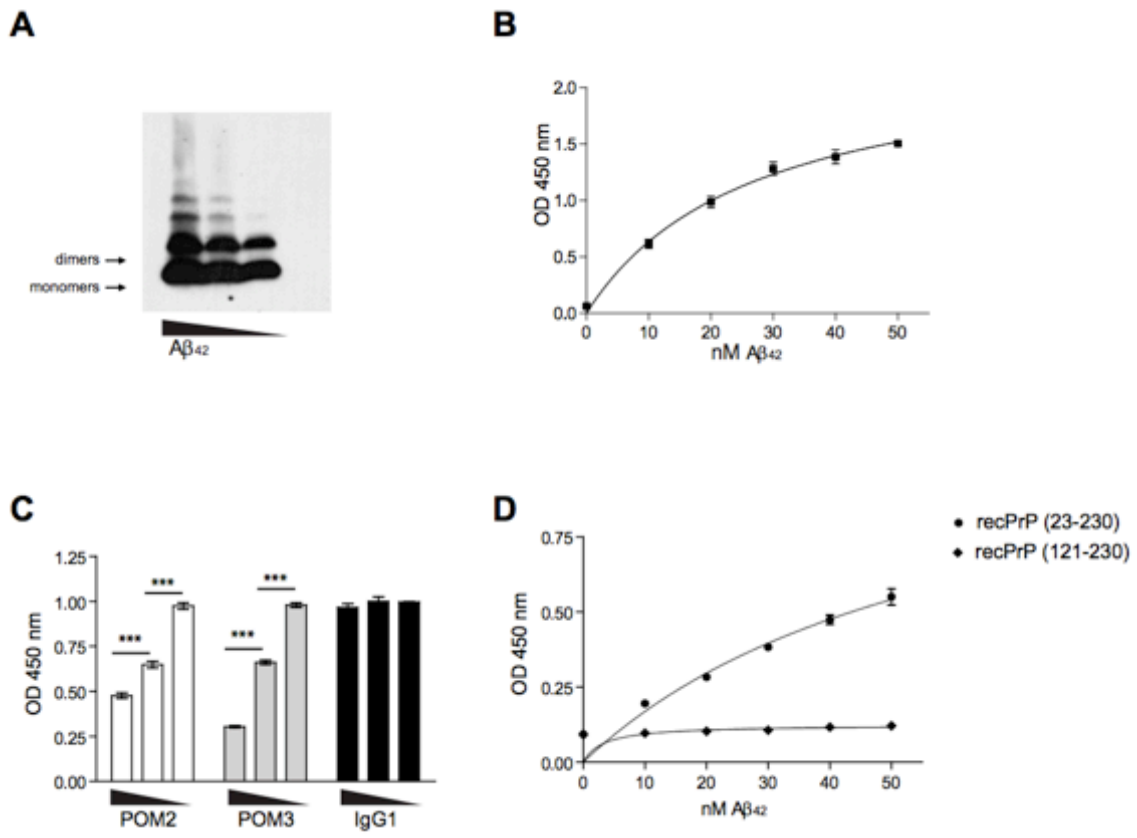


Figure S4. Recombinant PrP binds synthetic Aβ42 through its amino proximal domain.

(A) SDS-PAGE followed by protein blotting with an anti-human Aβ (6E10) antibody was used to characterize Aβ42 preparations (20, 10 or 5 ng of synthetic protein in each lane) for the experiments (B-D). (B) Titration of human Aβ42 onto immobilized recombinant PrP (recPrP23-231) obtained by ELISA showed binding of recPrP23-231 to Aβ42. (C) Binding of human Aβ42 to recPrP121-231 was reduced in presence of the POM2 and POM3 antibodies against the N-proximal region of PrPC. The epitope of POM2 lies within the octapeptide repeat region of PrPC, giving rise to four binding sites between residues 58 and 88. The epitope recognized by POM3 corresponds to amino acids 95-100 of mouse PrP. POM2, POM3, and IgG1 isotype control were utilized at different concentrations (100nM, 10nM, 1nM). Values are averages ± SD. Significance was determined by one-way ANOVA ***$p < 0.001$. (D) Comparison between the binding curves for human Aβ42 to immobilized recPrP23-231 or truncated recPrP121-231. Removal of the N-terminal region, as in recPrP121-231, prevented binding to Aβ42.

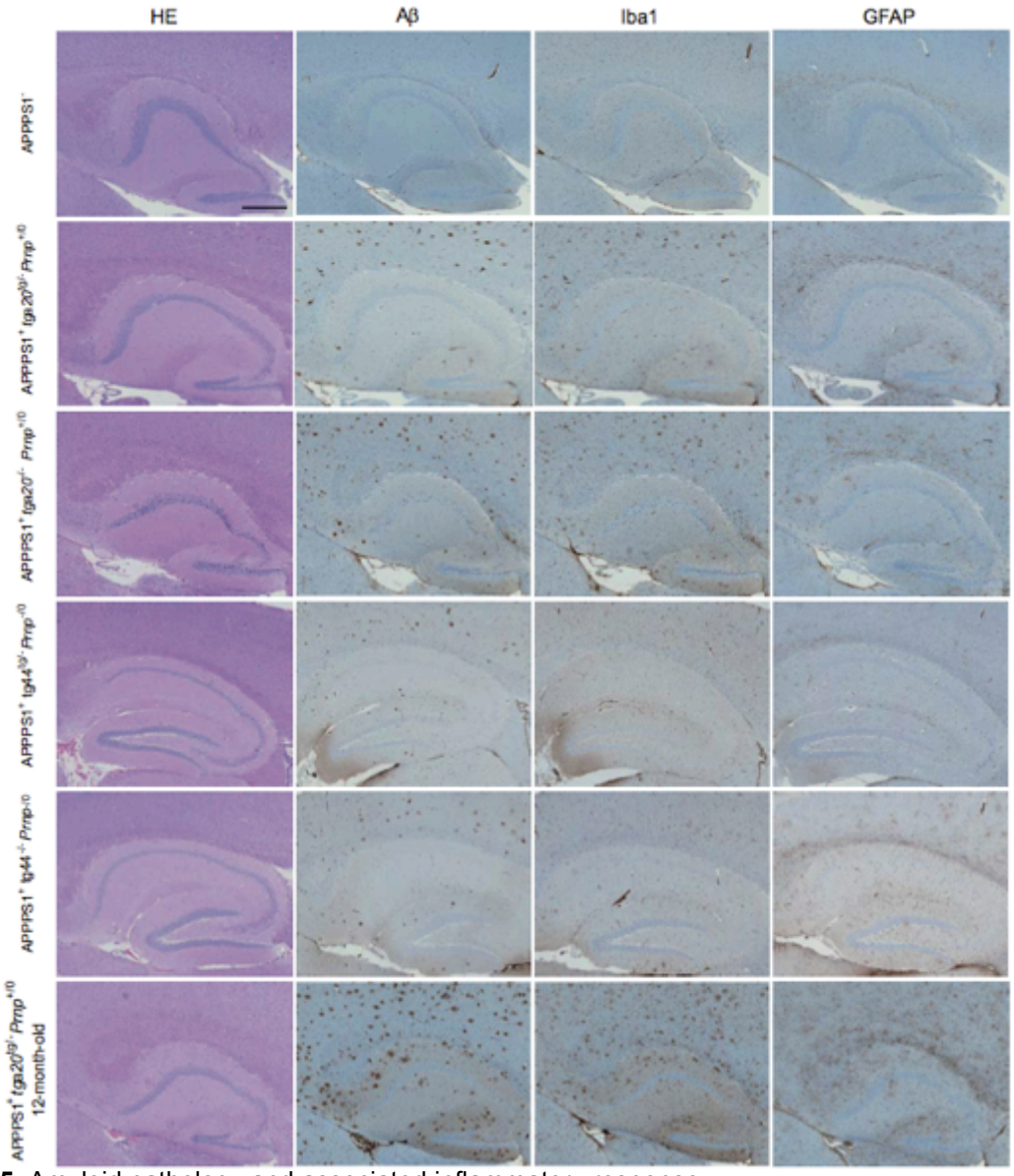


Figure S5. Amyloid pathology and associated inflammatory response.

Hippocampi of 4- month-old wild-type mice (1st row) and various APPPS1 mice (rows 2-5). The APPPS1 mice displayed similar degree of amyloid deposition, microglial activation, and astrocytosis. A 12- month-old APPPS1+tga20tg/-Prnp+/o mouse (bottom row) showed more pronounced amyloid deposition and associated inflammatory responses. HE: hematoxilin/eosin; Iba1: microglial marker; GFAP: glial fibrillary acidic protein, a marker of reactive astrocytes. Scale bar: 500 μm.

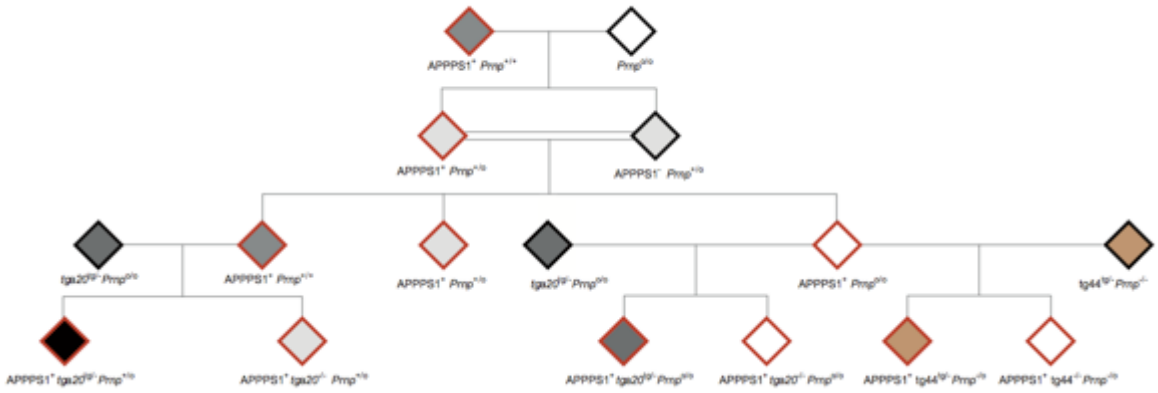


Figure S6. Crossing of genetically modified mice used in this study.

A representative pedigree showing intercrossing of several mutant mice is depicted. Grey scale indicates different levels of PrPC. Brown symbol: designate expression of anchorless, soluble PrP. The orange border denotes the presence of APP/PS1 transgenes. Parallel lines indicate brother- sister crossing. APPPS1- mice are not represented (with one exception) in the pedigree for clarity, but were included as controls in the actual experiments. Prnpo and Prnp- denote by convention the “Zurich-I” and “Edbg” knockout alleles of Prnp, respectively.

8.1.4 Material and Methods

Mice

To remove the prion protein locus (Prnp), Prnpo/o mice (Büeler et al, 1992) were crossed with APPPS1 mice (Radde et al, 2006). APPPS1+Prnpo/o or APPPS1 mice were then crossed with tga20tg/-Prnpo/o (Fischer et al, 1996) or tg44tg/-Prnp-/- mice (Chesebro et al, 2005) to generate the different APPPS1+ and APPPS1- littermate control mice (Table 1 and Fig S6). The genetic pattern of mouse strains was determined with a panel of 192 polymorphic microsatellites as described (Bremer et al, 2010). All mice were maintained under specific pathogen-free conditions. Housing and experimental protocols were in accordance with the Swiss Animal Welfare Law and in compliance with the regulations of the Cantonal Veterinary Office, Zurich.

Electrophysiology

Hippocampal slice preparation from male mice and fEPSPs recordings in the CA1 region were as described (Knobloch et al, 2007). The LTP induction protocol was considered successful, and entered in the analysis, only if a stable baseline for at least 10 min was achieved. To generate input–output curves, slices were prepared as above and stimulated every 20 sec with increasing intensity (from 0.0 to 0.1 mA in 0.01 mA increments) using a total of 10 stimuli. For comparing groups, potentiation of fEPSP slopes during the interval 10–25min post- tetanus was evaluated. Data points were normalized to the mean baseline value and expressed as mean ± SEM. All numbers in brackets indicate analyzed mice; 2–3 slices were typically analyzed for each mouse.

Tissue preparation

Brain fractionation was performed as described (Shankar et al, 2008) with modifications. Briefly, snap frozen forebrains were homogenized in ice-cold tris buffered saline (TBS), after centrifugation at 100,000 × g for 1 h the supernatant (called soluble fraction) was used to determine soluble Aβ42. The pellet was homogenized in phosphate buffered saline plus 0.5% 4-nonylphenyl-polyethylene glycol (NP40S), 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1- propanesulfonate (CHAPS) and spun at 16,000 × g for 30 min. The resultant supernatant was used to quantify APP, α-C terminal fragment (CTF) and β-CTF and the remaining pellet was solubilized in 70% formic acid and insoluble Aβ42 was measured after tris(hydroxymethyl)aminomethane (TRIS)-base neutralization.

Quantification of Aβ42 and PrPC

Levels of Aβ42 were assessed by sandwich enzyme-linked immunosorbent assay (ELISA; hAmyloid Aβ42, The Genetics Company) according to manufacturer's instructions. PrPC concentration was determined by sandwich ELISA as described (Polymenidou et al, 2008).

Immunoblotting

To determine APP and CTFs levels, 20 mg of proteins were separated by electrophoresis on a 4–12% polyacrylamide gel. Primary antibodies were: anti-APP C-terminal (Sigma) recognizing both mouse and human APP and CTFs; anti-actin (Chemicon). Protein bands were detected by adding SuperSignal West

Pico Chemiluminescent Substrate (Pierce) and exposing the blot in a Stella detector (Raytest). Chemiluminescence quantification was performed by TINA software.

In vitro binding assay

Binding of synthetic human Aβ42 (Bachem AG) to immobilized recombinant PrP (Zahn et al, 1997) was analyzed by ELISA. Recombinant PrP (recPrP23–231 or recPrP121–231) was immobilized overnight at 48C on 96-well microtiter plates. Varying concentrations of synthetic human Aβ42 were added to wells and incubated for 1 h. Bound proteins were detected by incubation with 6E10 antibody (Covance) followed by horseradish peroxidase-conjugated antimouse IgG1. Absorbance was measured at 450 nm. For Western blot analysis various concentrations of Aβ42 were incubated in the same conditions, followed by Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotting with 6E10 antibody. Binding of human Aβ42 (25 nM) to recPrP23–231 was assessed also in presence of decadic dilutions (100, 10 and 1nM) of anti-PrP antibodies (Polymenidou et al, 2008).

Histological analyses

Brains were removed and fixed in 4% formaldehyde in phosphate buffered saline, pH 7.5, paraffin embedded and cut into 2–4mm sections. Sections were stained with hematoxylin–eosin (HE) or antibodies against glial fibrillary acidic protein (GFAP) (DAKO), ionized calcium binding adapter molecule 1 (Iba1; WAKO) and Aβ (4G8; Signet).

Statistical analyses

Statistical significance was determined according to one-way ANOVA followed by Tukey's post-test for multiple comparison, unpaired Student's t-test and Mann–Whitney test using Prism software (GraphPad Software). Error bars in the graphs and numbers following the ± sign denote standard errors of the mean unless otherwise indicated.

Author contributions

A.M.C. designed the study, organized and maintained the mouse colony, performed biochemical and histologic analyses, analyzed the data and cowrote the paper; M.F. performed electrophysiology experiments, analyzed the data and cowrote the paper; M.N. helped in organizing and maintaining the mouse colony, performed genetic analyses, analyzed the data and cowrote the paper; O.M. performed electrophysiology experiments and analyzed the data; R.M. performed biochem- ical experiments; J.F. performed biochemical experiments; I.M.M. supervised electrophysiology experiments, analyzed the data and wrote the paper; A.A. designed and coordinated the study, supervised biochemical, genetic and histologic analyses, analyzed the data and wrote the paper.

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Supporting information is available at EMBO Molecular Medicine Online.

The authors declare that they have no conflict of interest.

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8.2 Side Project 2

Pathological brain plasticity and cognition in the offspring of fathers subjected to traumatic early life stress

Johannes Bohacek[1], Melissa Farinelli[1], Osvaldo Mirante[1], Guyllaume Coiret[1], Guido Steiner[3], Martin Ebeling[3], Gonzalo Durán-Pacheco[3], A. Leonardo Iniguez[2], Francesca Manuella[1], Katharina Gapp[1], Jean-Luc Moreau[3], Isabelle M. Mansuy[1]

[1] Brain Research Institute, Neuroscience Center Zürich, University of Zurich / ETH Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

[2] Roche NimbleGen, Inc., 504 S. Rosa Road, Madison WI 53719, USA

[3] Pharma Division, Hoffmann-La Roche, Basel, Switzerland

Corresponding author: Isabelle Mansuy Tel: +41 44 635 33 60, Fax: +41 44 635 33 03, mansuy@hifo.uzh.ch

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Attribution of data: I have performed the electrophysiological experiments for figures 1A, 1B, S2 and S3, as well as analyzed the results. I also contributed to the writing of the Materials and Methods.

8.2.1 Abstract

Traumatic stress in early life increases the risk for cognitive and neuropsychiatric disorders across life. Through epigenetic mechanisms, such experiences can also impact the progeny even if not directly exposed to stress. Here, we report in mice that the adult offspring of stressed males have altered molecular pathways necessary for neuronal signaling, and dysfunctional synaptic plasticity. Specifically, long-term potentiation is abolished and long-term depression is enhanced in the hippocampus. These defects are associated with impaired long-term memory in both the offspring and the stressed fathers. The brain-specific gamma isoform of protein kinase C (*Prkcc*) is one of the affected signaling components in the hippocampus. Its expression is reduced in the offspring, and DNA methylation at its promoter is altered both in the hippocampus of the offspring and the sperm of fathers. These results suggest that postnatal traumatic stress in males can affect brain plasticity and cognitive functions in the progeny when adult, likely through epigenetic changes in the male germline.

8.2.2 Introduction

Early life stress resulting from emotional and physical neglect or abuse in childhood is a major risk factor for the development of psychiatric conditions such as major depression, bipolar disorder, schizophrenia and post-traumatic stress disorder [269]. Considering that about 700,000 children are victims of neglect or abuse each year in the United States alone [270,271], the long-term consequences of such trauma place a heavy burden on society and the healthcare system. Recent studies in mice have demonstrated that exposure of male pups to traumatic stress involving repeated episodes of unpredictable maternal separation combined with maternal stress (MSUS), leads to depressive-like behaviors, altered risk assessment and impaired social interactions in adulthood across several generations [263,265,272]. This suggests that paternal trauma is a risk factor for the development of behavioral disorders in the progeny [262,273]. In addition to affective and emotional disorders, cognitive dysfunctions are also common to many stress-induced conditions [271,274-276], in part because the hippocampus, a brain region critical for learning and memory formation, is an important component of stress response pathways [277,278]. Animal models have established that early trauma can have lifelong negative consequences on cognitive performance and synaptic plasticity in the hippocampus of exposed animals [279-281], but the impact on the offspring has not yet been carefully assessed.

Using the MSUS mouse model, we conducted an unbiased, genome-wide analysis of gene expression in the adult hippocampus following postnatal traumatic stress, and determined whether gene networks are affected in the offspring of the stressed males. Here, we show that the offspring of males exposed to MSUS have widespread alterations in gene expression in the hippocampus, specifically in molecular networks implicated in synaptic plasticity. Further, the animals have a dramatic shift in functional synaptic plasticity, in particular abolished long-term potentiation (LTP) and enhanced long-term depression (LTD). This shift is accompanied by impaired hippocampus-dependent long-term memory. We identify the brain-specific gamma subunit of protein kinase C (*Prkcc*), a gene implicated in synaptic plasticity and memory performance [282,283], as potential molecular target. The expression of *Prkcc* is decreased in the hippocampus of the offspring, and DNA methylation is reduced at a specific transcription factor binding site in the promoter region both in the brain of the offspring and the sperm of fathers.

8.2.3 Results

To test the effects of early life stress across generations, we subjected newborn mouse pups (F1) to MSUS for two weeks, and then bred the males when adult to naïve (non-stressed) wild-type females to generate F2 progeny. To validate the efficacy of the MSUS manipulation, we examined depressive-like behaviors on a forced swim test. Adult F1 MSUS males and the F2 female offspring spent more time floating than controls (F1: controls: 48.3±6.2; MSUS: 71.0±6.6 sec, $t(60)=2.5$, $p=0.015$. F2: controls: 46.1±5.6; MSUS: 77.2±7.6 sec; $t(28)=3.30$, $p=0.003$, Supplementary Figure 1), confirming previous findings that MSUS triggers the expression of depressive-like symptoms across generations [263-265,272].

Once depressive symptoms were confirmed in MSUS animals, we conducted genome-wide DNA microarrays analysis in adult F2 females at rest (baseline resting condition) to determine which molecular pathways are affected by MSUS. In the hippocampus, a brain area implicated in depression [284], ANOVA revealed that 156 genes were differentially regulated by at least 1.2-fold (data available through GEO, accession number GSE47848) in F2 MSUS mice. However, because multiple testing correction (FDR method) proved too stringent for the dataset (see Methods), we used a gene set-based approach to determine whether gene expression was altered at the network level [285]. Gene set enrichment analyses (GSEA) identified 49 up-regulated and 30 down-regulated molecular pathways in F2 MSUS hippocampus compared to controls (for a complete list, see Supplementary Table 1). Notably, the down-regulated

pathways contained several partially overlapping components critical for excitatory synaptic transmission (i.e. NMDA receptor-dependent signaling), neuronal plasticity, and memory formation (Table 1, top).

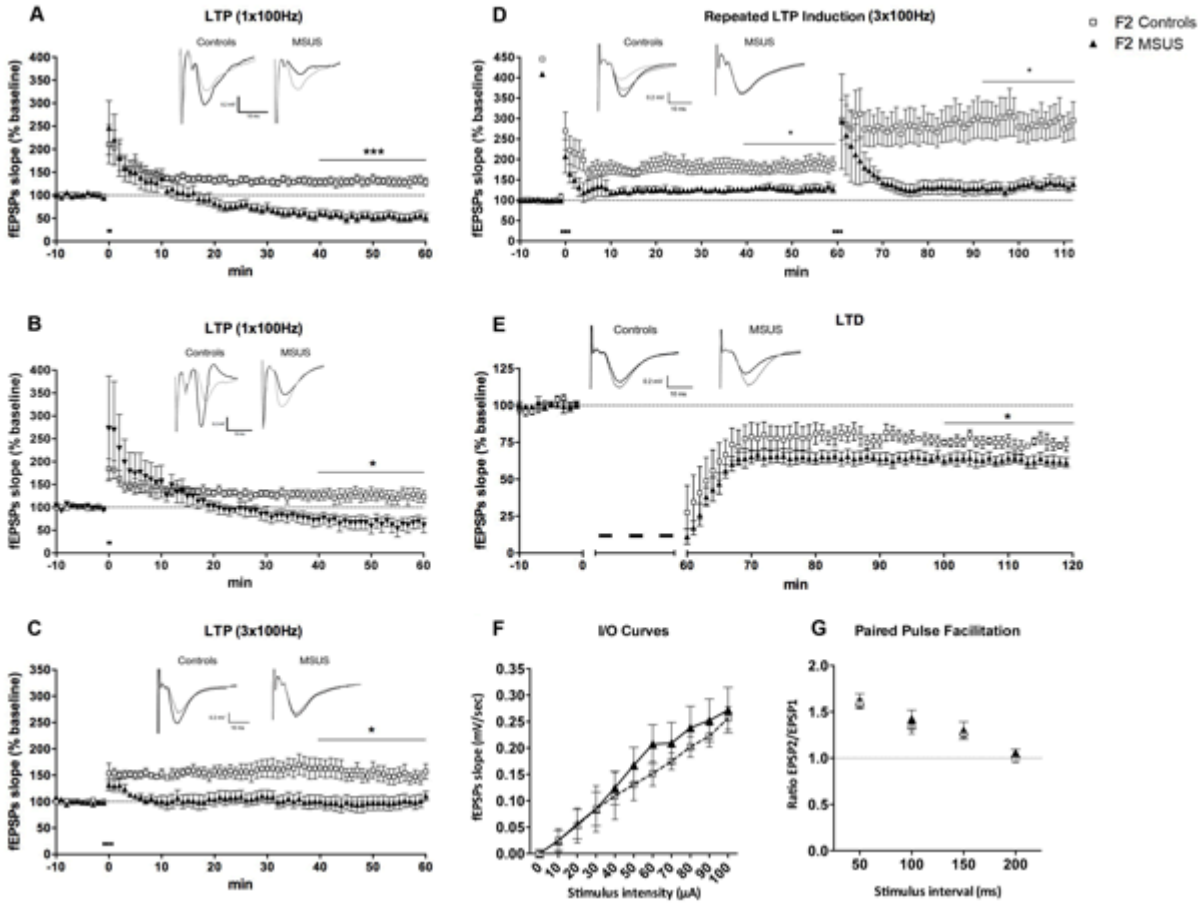

Figure 1. The offspring of MSUS males have altered hippocampal LTP and LTD.
(A) Hippocampal LTP induced by 1x100Hz stimulation is impaired in F2 MSUS males (n=5 mice) compared to controls (n=4 mice). (B) A similar impairment is observed in F2 MSUS females (n=5 mice) compared to controls (n=5 mice). (C) LTP induced by 3x100Hz stimulation leads to impaired LTP in F2 MSUS mice (n=6 mice) compared to controls (n=6 mice). (D) After repeated 3x100Hz stimulation, the LTP impairment persists in MSUS mice (n=4 mice) compared to controls (n=6 mice). (E) LTD is enhanced in F2 MSUS mice (n=4 mice) compared to controls (n=4 mice). (F) Input-output curves are comparable in F2 MSUS mice (n=6 mice) and controls (n=6 mice). (G) Paired pulse facilitation is comparable in F2 MSUS mice (n=5 mice) and controls (n=6 mice). Thick black bars schematically indicate the LTP/LTD stimulation protocol. Horizontal bars indicate the last 20 min of recording used for statistical analyses. Inset traces show a

representative fEPSP from each group, gray traces represent the average of baseline recording before stimulation, black traces show the average over the last 20 min of post-stimulation recording. Data are mean ± s.e.m. ***=p<0.001, **=p<0.01, *=p<0.05

MSUS was previously shown to alter behavioral responses in stressful and aversive conditions in adult animals across generations [263-265,272]. Thus, we postulated that differences in gene expression between MSUS and control mice may be more pronounced in response to a stress challenge. We repeated the DNA microarray analyses in F2 animals 45 minutes after exposure to a session of forced swim, an acute form of stress that activates gene expression in the hippocampus [286]. Using the same statistical criteria as above, 1,782 genes with differential expression could be identified in F2 MSUS mice after acute stress, representing a 10-fold increase compared to baseline resting conditions. GSEA identified 25 gene pathways significantly down-regulated in MSUS mice, but no up-regulated pathway (Supplementary Table 1). Notably, the down-regulated pathways included NMDA receptor-dependent signaling, synaptic calcium signaling and synaptic plasticity networks, which are similar to those identified in resting conditions (Table 1). Overall, these results show that plasticity-related gene networks are compromised in the offspring of MSUS mice, both at rest and after stress.

Brain plasticity pathways down-regulated in F2 MSUS mice - Baseline resting condition

Rank	Gene set name	Size	NES	Nom. p-val	FDR q-val
5	Reelin signaling pathway – NCI / Nature pathway	28	-1.799	0.0009	0.1130
6	Unblocking of NMDA receptor glutamate binding and activation – Reactome pathway	15	-1.745	0.0026	0.2058
8	Synaptic transmission – Reactome pathway	150	-1.585	0.0012	0.2172
14	CREB phosphorylation through the activation of CaMKII – Reactome pathway	13	- 1.697	0.0055	0.2318
17	CREB phosphorylation through the activation of RAS – Reactome pathway	23	-1.547	0.0228	0.2394
19	Activation of NMDA receptor upon glutamate binding and postsynaptic events – Reactome pathway	33	-1.567	0.0137	0.2395
20	RAS activation upon Ca^{2+} influx through NMDA receptor – Reactome pathway	15	-1.673	0.0075	0.2400
30	Glutamate binding – Activation of AMPA receptors and synaptic plasticity – Reactome pathway	28	-1.677	0.0058	0.2479

Brain plasticity pathways down-regulated in F2 MSUS mice - Acute stress condition

Rank	Gene set name	Size	NES	Nom. p-val	FDR q-val
1	DARPP 32 events - Reactome pathway	23	-2.356	0.0000	0.0082
3	RAS activation upon Ca^{2+} influx through NMDA receptor – Reactome pathway	14	-2.169	0.0006	0.0278
4	GABA synthesis, release, reuptake and degradation – Reactome pathway	19	-2.101	0.0000	0.0459
8	CREB phosphorylation through the activation of CaMKII – Reactome pathway	13	-1.930	0.0036	0.1428
13	AKT phosphorylates targets in the cytosol – Reactome pathway	13	-1.867	0.0073	0.1556
20	PI3K-AKT activation – Reactome pathway	36	-1.779	0.0024	0.2050
21	Transmission across chemical synapses – Reactome pathway	173	-1.769	0.0000	0.2095

Table 1. Pathways identified as down-regulated in the hippocampus of F2 MSUS mice relative to controls by GSEA.

Pathways involved in synaptic plasticity are ranked by false discovery rate (FDR) adjusted p-value. Rank = Rank of the pathway in the complete set of down-regulated pathways. Size = Number of genes in each pathway. NES = normalized enrichment score. Nom. p-val = Nominal (unadjusted) p value.

An alteration of signaling pathways can dramatically affect neuronal and network functions. The broad and coordinated molecular changes detected in plasticity-related pathways in F2 MSUS mice may therefore have important functional consequences. We tested this possibility by examining synaptic plasticity in different brain regions in adult F2 mice. In hippocampus area CA1, long-term potentiation (LTP) induced by one train of 100Hz stimulation, a form of synaptic strengthening, was abolished in MSUS slices and instead, synaptic depression was induced. This effect persisted through recording and was the most pronounced 40-60min after the tetanus in both, males and females (Males: controls, 135.3 ± 12.1%; MSUS: 43.05 ± 4.6%; $t(7)=7.86$, $p<0.001$. Females: controls, 126.0 ± 13.6%; MSUS: 65.81 ± 14.4%; $t(8)=3.04$, $p=0.016$, Figure 1). Two-pathway recordings confirmed that the observed depression in F2 MSUS mice was not due to a reduced viability of slices, since a stable response could be induced by basal stimulation in a non-tetanized pathway (Supplementary Figure S2). We next examined whether a stronger stimulation could elicit LTP in MSUS slices and used three trains of 100Hz tetanus, a stimulation known to produce late phase LTP [287]. Like 1-train LTP, three-train LTP was abolished in F2 MSUS slices compared to control slices (40-60 min post tetanus, controls: 153.3 ± 13.4%; MSUS: 98.0 ± 12.7%; $t(10)=2.98$, $p=0.013$; Figure 1C), even when the stimulation was repeated twice (LTP after 1st tetanus: controls 183 ± 15.8%; MSUS: 128.3 ± 7.5%, $t(8)=2.66$; $p=0.029$. LTP after 2nd tetanus: controls 290.9 ±

40.79%; MSUS: 134.3 ± 13.4%; t(8)=3.01; p=0.017, Figure 1F). We next examined LTD, a form of synaptic weakening induced by low frequency stimulation. In hippocampus area CA1, LTD was stronger in F2 MSUS slices than in control slices (controls: 74.9 ± 2.9%; MSUS: 63.0 ± 3.7%; t(6)=2.50, p=0.046; Figure 1G). The changes in plasticity in F2 MSUS hippocampus were not due to any gross alteration in basal synaptic transmission since input-output curves were similar in control and MSUS slices (Figure 1D). They were also not due to any major alteration in neurotransmitter release since paired pulse facilitation (PPF), a short-term form of presynaptic plasticity [288], was comparable in control and MSUS slices (Figure 1E). We then tested if plasticity was disrupted in other brain areas and examined LTP in the lateral amygdala (LA), a part of the limbic system implicated in fear memory. Stimulation of the thalamic or cortical pathways in LA [184] showed that LTP was impaired in both pathways in MSUS slices (thalamic pathway: controls: 181.1 ± 15.3%; MSUS: 115.1 ± 19.4%; t(11)=2.61, p=0.024; cortical pathway: controls: 205.6 ± 52.9%; MSUS: 110.8 ± 12.7%; t(10)=2.05, p=0.068, Supplementary Figure S3). Together, these results indicate a global alteration of synaptic plasticity in several brain areas in the F2 offspring, with a shift in plasticity towards synaptic depression in the adult hippocampus.

To determine if the LTP impairment in the offspring was inherited from the fathers, we examined LTP in the hippocampus of F1 MSUS males. Three-train LTP was abolished in F1 MSUS males (controls: 193.5 ± 16.7%; MSUS: 112.7 ± 13.1%; t(9)=3.87, p=0.004; Figure 2A), similarly to that in F2 offspring,

suggesting that the LTP defect was transmitted from fathers to offspring. We then tested if transmission depends on maternal care by conducting cross-fostering. When adult, F2 MSUS pups raised by control dams (F2 MSUS-CD) had impaired three-train LTP, while F2 control pups raised by dams mated to MSUS males (F2 controls-MD) had normal LTP (MSUS-CD: 117.6 ± 7.2%; t(16)=4.31, control-MD: 181.8 ± 12.0%; p<0.001, Figure 2B). These results demonstrate that the negative effects of paternal stress on LTP are transmitted to the offspring via a route independent of maternal care, likely involving the male germline [264,272,273]. Finally, we tested whether the LTP defect could also be transmitted to the F3 offspring and for this, bred F2 males to naïve females. Three-train LTP was robust in both controls and MSUS F3 mice (controls: 214.7 ± 13.6%; MSUS: 177.3 ± 18.1%; t(11)=1.604, p=0.137, Figure 2C), indicating that MSUS affects plasticity in directly exposed mice (F1) and their progeny (F2), but not in the following generation (F3).

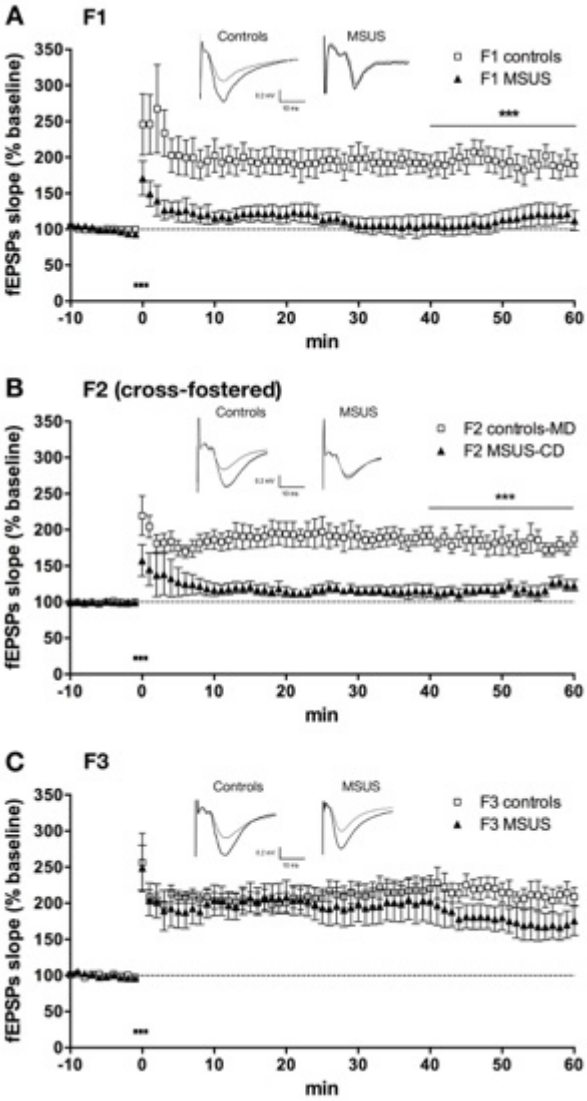

Figure 2. Impaired LTP in MSUS mice across generations.
(A) LTP impairment is observed in F1 MSUS mice (n=6 mice) compared to controls (n=6 mice). (B) A similar LTP impairment is observed in MSUS offspring (F2 generation) raised by control dams after cross-fostering (MSUS-CD; n=8 mice). Control offspring raised by dams previously mated with MSUS males have intact hippocampal LTP (control-MD; n=10 mice). (C) In the F3 generation, LTP can be similarly induced in MSUS mice (n=7 mice) and controls (n=6 mice). Horizontal bars indicate the last 20 min of recording used for statistical analyses. Inset traces show a typical fEPSP from each group, gray traces represent the average of baseline recording before stimulation, black traces show the average over the last 20 min of post-stimulation recording. Data are mean ± s.e.m. ***=p<0.001

Synaptic plasticity in the adult brain is important for memory formation, therefore we examined memory performance in the animals. We tested contextual fear memory using a paradigm in which a novel context is associated with an aversive stimulus (mild foot-shock). While baseline freezing was similar in control and MSUS mice from F1, F2 or F3 generations (Figure 3, left), F1 MSUS mice spent

significantly less time freezing than controls 24 hours after fear conditioning ($t(18)=2.52$, $p=0.021$, Figure 3A). F2 MSUS offspring had a similar lower freezing 24 hours after conditioning ($t(22)=2.66$, $p=0.014$, Figure 3B). In contrast, freezing was normal in F3 MSUS offspring ($t(28)=0.82$, $p=0.417$; Figure 3C), suggesting that MSUS impairs contextual fear memory in animals directly exposed to MSUS (F1) and their offspring (F2), but not in the following generation (F3).

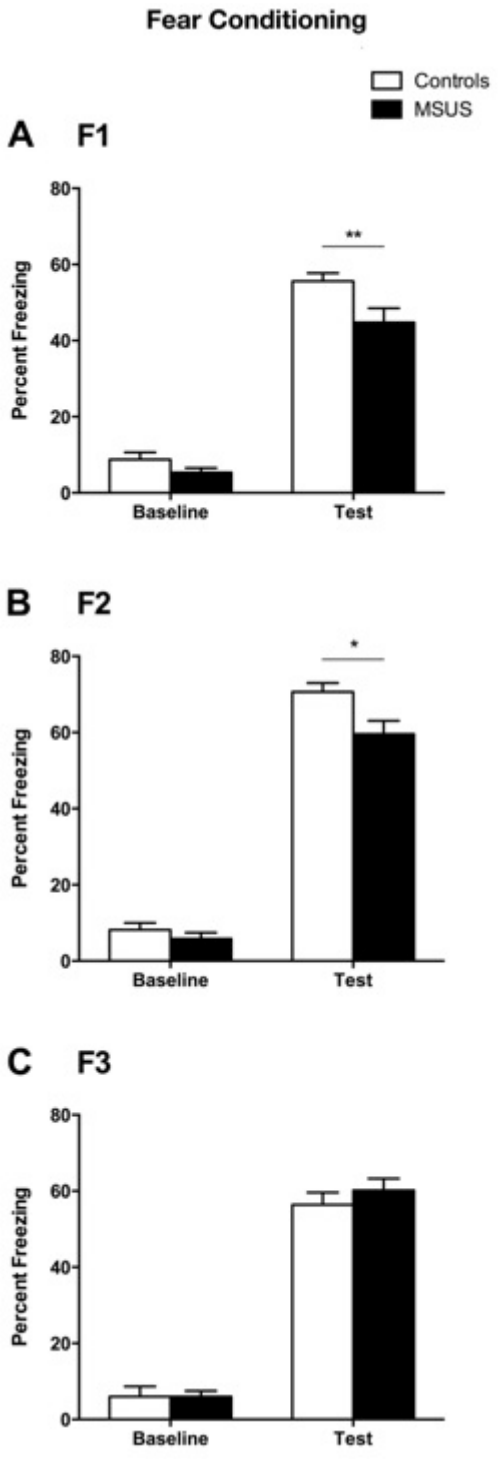

Figure 3. Impaired fear memory in MSUS mice across generations.

Twenty-four hours after contextual fear conditioning, F1 (A) and F2 (B) MSUS mice spend less time freezing than controls (F1: controls, n=10; MSUS, n=13. F2: controls, n=12; MSUS, n=12) but not F3 MSUS mice (controls, n=12; MSUS, n=18). Left bars show baseline freezing before

delivery of the foot-shock. Data are mean ± s.e.m. **=p<0.01, *=p<0.05

To examine if MSUS affects other forms of memory, we tested the animals on an object recognition task. The animals were trained to memorize several objects in a familiar arena, then their memory for the objects was evaluated 2.5 or 24 hours later [146]. During training, F1 control and MSUS mice explored all objects equally, suggesting similar acquisition (data not shown). When tested 2.5 hours later, both groups spent more time exploring a novel object than the familiar objects (Controls: $t(7)=4.11$, $p=0.004$; MSUS: $t(12)=3.71$, $p=0.003$; Figure 4A), indicating normal object memory. However, 24 hours after training, while control mice spent more time exploring the novel object ($t(9)=4.43$, $p=0.002$), F2 MSUS mice could not discriminate it from the familiar objects ($t(10)=1.60$, $p=0.14$, Figure 4B), indicating impaired long-term object memory. Similarly to F1 animals, F2 MSUS mice had normal object memory 2.5 hours after training (Controls: $t(15)=3.90$, $p=0.003$; MSUS: $t(13)=3.46$, $p=0.004$; Figure 4C), but impaired memory after 24 hours (Controls: $t(15)=3.90$, $p=0.001$); MSUS: $t(14)=1.65$, $p=0.12$, Figure 4D). Together, these data suggest that different forms of long-term memory are impaired by MSUS across generations.

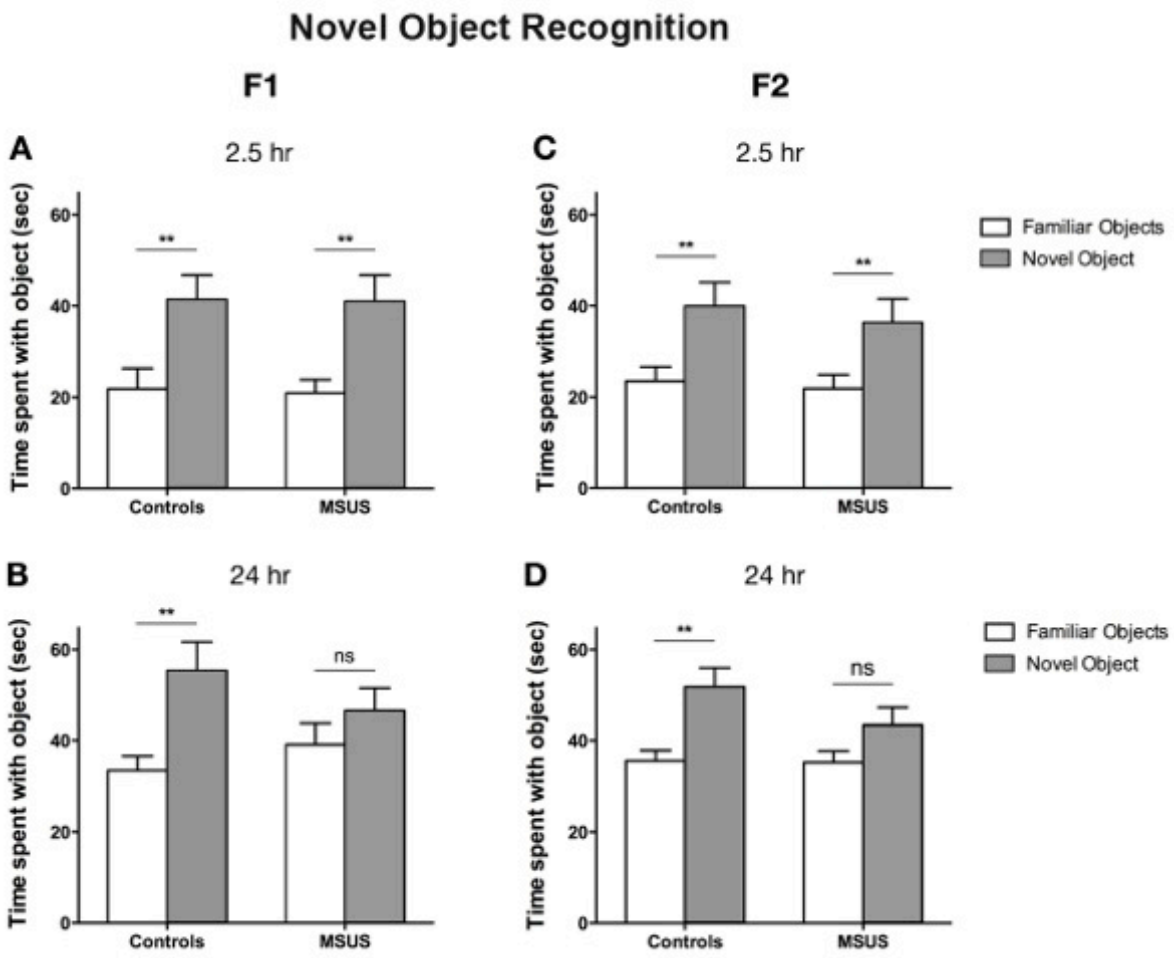

Figure 4. Impaired object recognition memory in MSUS mice across generations. Following 2.5 hrs after training, control mice and MSUS mice spend more time exploring the novel object than the familiar objects, both (A) in F1 animals (controls: n=10; MSUS: n=13), and (C) the F2 offspring (controls: n=15; MSUS: n=15). Twenty-four hrs after training, control mice spend more time exploring the novel object, but MSUS mice do not discriminate between novel and familiar objects, both (B) in F1 animals (controls: n=10; MSUS: n=11) and (D) the F2 offspring (controls: n=16; MSUS: n=15). Data are mean ± s.e.m. **=p<0.01, *=p<0.05

In light of the impaired plasticity in MSUS animals, we next examined potential molecular targets in plasticity pathways found to be altered in the DNA microarray analyses. Using RT-qPCR, we observed that several genes critical for synaptic plasticity were downregulated in the hippocampus of F2 MSUS mice,

including protein kinase C gamma (*Prkcc*; t(13)=3.03, p=0.009), NR1 subunit of the NMDA receptor (*Grin1*; t(13)=2.46; p=0.028), metabotropic glutamate receptor 1 (*Grm1*; t(13)=2.26, p=0.044), calcium/calmodulin-dependent protein kinase II alpha (*Camk2a*; t(13)=1.88, p=0.082) and ionotropic glutamate receptor AMPA3 (*Gria3*; t(13)=1.85, p=0.088) (Figure 5A, left panel). Several housekeeping genes including *Hprt, Actb* and *Gapdh* were not altered (Figure 5A, right panel), suggesting pathway-specific suppression of gene expression. To investigate the potential mechanisms underlying the altered Prkcc expression, we examined whether DNA methylation, an epigenetic mode of gene regulation previously implicated in the expression and transmission of the effects of MSUS [264,272], is affected. We focused on DNA methylation at the *Prkcc* gene because this neuron-specific isoform of PKC is involved in LTP induction and memory processes [282,283]. Using bisulfite pyrosequencing, we quantified DNA methylation in a proximal promoter region of *Prkcc* that carries transcription factor binding sites sufficient for promoter activity [289,290] (Figure 5B). DNA methylation was overall low across CpGs contained in this region, except at CpG 6, an Sp1 binding site that can bind the transcriptional repressor and/or activator Sp1 and Sp3 with equal affinity [291], in control samples. At this site, DNA methylation was significantly reduced in F2 MSUS hippocampus compared to controls (t(10)=3.30; p=0.008; Figure 5C). Likewise, in sperm samples of F1 mice, DNA methylation was low at most CpGs except at CpG 6, and like in the hippocampus, it was significantly downregulated at this site in MSUS samples (t(12)=2.34, p=0.038, Figure 5D).

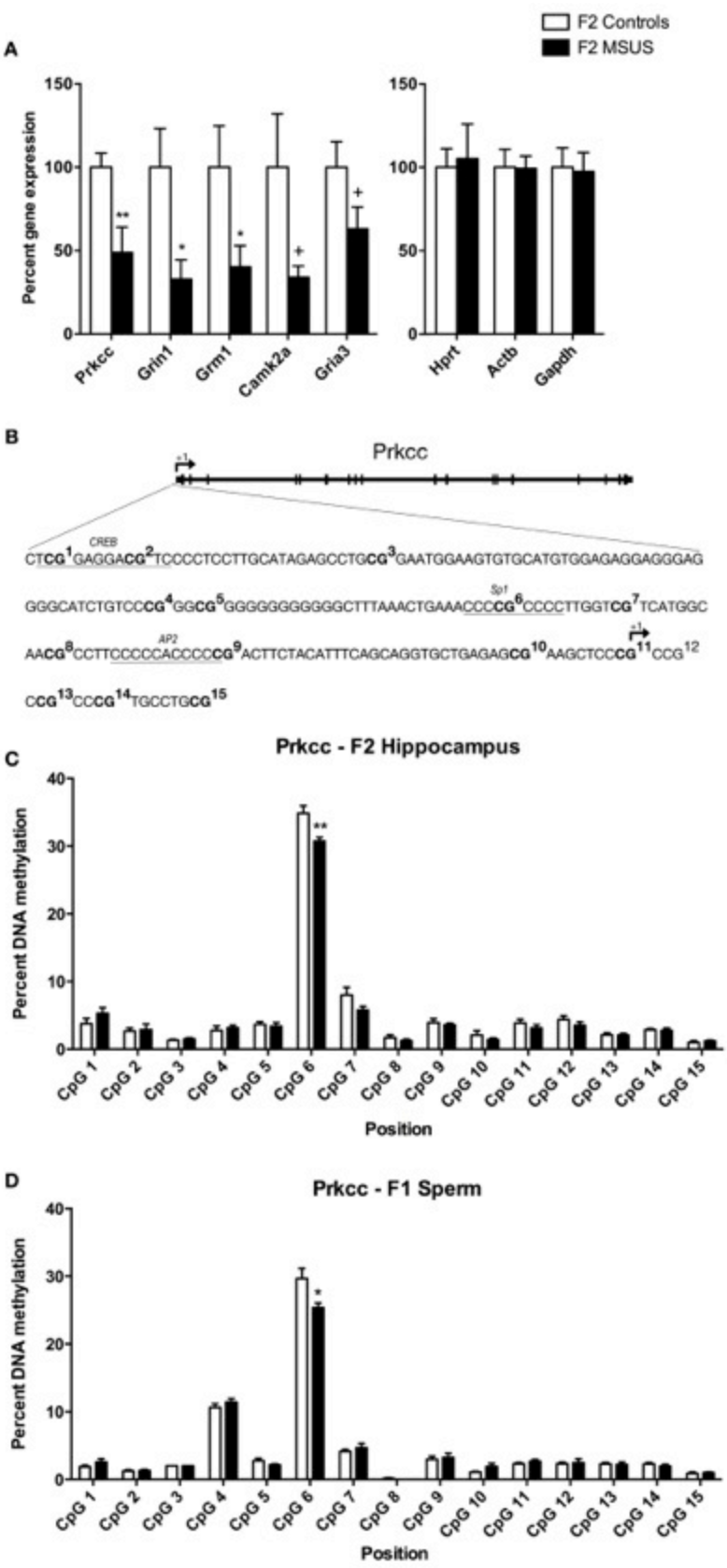

Figure 5. MSUS alters *Prkcc* expression and DNA methylation in the *Prkcc* promoter in F1 sperm and F2 brain.

(A) Left: RT-qPCR confirms decreased expression of genes related to synaptic plasticity and memory in the hippocampus of F2 MSUS mice (n=8) compared to controls (n=8). Right: No group differences are detected for housekeeping genes. (B) Schematic representation of the *Prkcc* promoter region analyzed by pyrosequencing including the transcription start site (+1) and the binding site for CREB, Sp1 and AP2. Numbers represent individual CpG sites analyzed for DNA methylation (CpG 1-15). (C) DNA methylation is reduced at CpG 6 in the hippocampus of F2 MSUS mice compared to controls (n=6/group). (D) DNA methylation is reduced at CpG 6 in sperm of F1 MSUS mice (n=7) compared to controls (n=8).

8.2.4 Discussion

Using a model of chronic and unpredictable traumatic stress in early life in mice, we demonstrate for the first time that such stress dramatically alters synaptic plasticity in different brain areas and impairs long-term memory in both, the animals directly exposed to stress and in their offspring. These defects are associated with changes in several molecular pathways involved in plasticity and memory, and with specific components of these pathways such as *Prkcc*. We show that *Prkcc* expression is altered by traumatic stress in the hippocampus of the offspring and that DNA methylation in *Prkcc* promoter is reduced in both the hippocampus of the offspring, and the sperm of fathers.

Early life stress is known to impair neuronal plasticity and cognitive functions during adulthood in rodents and humans [280,292,293], in part through perturbations in glutamatergic pathways downstream of NMDA receptors [294-296]. However, transgenerational effects on synaptic plasticity have not been examined so far. Our findings that several glutamatergic signaling networks are altered in the hippocampus of the progeny of MSUS males demonstrate that the impact of stress on plasticity pathways is specific and transgenerational. Two of the molecular pathways suppressed by MSUS both at rest and following acute stress, are “calcium-mediated RAS activation through NMDA receptors”, and “CaMKII-dependent CREB phosphorylation” pathways. These pathways are critical for the induction and the maintenance of LTP [297-299], and may therefore underlie the shift in hippocampal plasticity of the offspring. Such a shift was previously reported in response to acute stress [300-302] or chronic social defeat

stress [303], but our data newly show that it occurs also in the progeny of animals subjected to stress. It is reminiscent of the synaptic modification model of homeostasis that proposes that neuronal networks can adapt to repeated strengthening or weakening of synapses [303-306]. In turn, it suggests that early life stress may modulate the synaptic range rather than causing a mere failure in synaptic strengthening, and may explain why the MSUS offspring have a relatively mild memory deficit despite a total absence of LTP. The altered expression of plasticity-related genes in MSUS offspring including *Prkcc*, likely underlies these defects. Indeed, knockout mice deficient for *Prkcc* have a phenotype remarkably similar to MSUS mice; they lack LTP [282,283] and have mild memory impairments [282,283]. However, *Prkcc* is only one of several affected targets in MSUS animals, and the phenotype likely results from the combined action of all altered genes. Network-wide expression changes were recently reported in the paraventricular nucleus (PVN) and the bed nucleus of stria terminalis in the offspring of stressed fathers [307]. Together, these data suggest that broad transcriptomic changes occur in the offspring of stressed fathers in several brain regions and may subserve different functions.

We have previously described transgenerational impairments in social recognition memory following MSUS [263]. The current results extend these findings by showing that cognitive functions are also affected. Interestingly, while our data show the negative impact of early traumatic stress on both plasticity and cognitive functions across generations, another study showed that stimulating environmental conditions can positively modulate plasticity and cognition [308]. If

mice were transiently exposed to enriched environmental conditions early in life, their offspring had enhanced LTP and better contextual fear memory. These effects persisted through cross-fostering. Although the effect of enrichment was transmitted by females and not males, this suggests that plasticity and memory are sensitive to ancestral experience and can be impaired or enhanced in the offspring, depending on the environment encountered by the parents. In this context, our study shows that both females and males have altered plasticity, while previous studies have reported sex-specific effects in the offspring [263-265,272,291,309]. These results suggest that MSUS has a global impact in the offspring independent of sex programming.

Paternal effects of environmental factors including stress [264,272,310,311], endocrine disruptors [312,313], diet [291,309,314] and drugs of abuse [315] on the offspring have been documented but the mechanisms underlying transmission remain poorly understood. In our model, since the transmitting males contribute only their germ cells and are never in contact with the offspring [273], transmission most likely implicates epigenetic mechanisms in the germline. The altered DNA methylation in MSUS fathers' sperm i.e. at the *Prkcc* promoter, provides evidence that DNA methylation in germ cells is associated with transmission. This complements our previous demonstration of DNA hypo- or hyper-methylation at several loci in MSUS sperm and the brain of F2 offspring [264,272]. Such alterations may be maintained or relayed by other mechanisms in the developing embryo (which undergoes widespread demethylation) and contribute to the adult phenotypes. How DNA methylation is modified by early life experiences in sperm cells, and

how changes are targeted to specific loci remain unknown. Although male germ cells are the primary career, maternal care may also contribute to the transmission of paternal effects since females can adjust their level of care depending on the fitness and attractiveness of their mate [316,317]. This possibility is however excluded for the MSUS model since the impairments (LTP) persist after cross-fostering. Interestingly, while emotional reactivity, depressive-like behaviors and social behaviors due to MSUS are transmitted down to the F3 generation [263,264,272], the LTP and memory impairments are transmitted only to F2 and do not affect F3 animals (Figures 2 and 3). This suggests that different mechanisms may be recruited for transmission, some that persist across multiple generations and some that are more transient [262,318]. More work is however needed to identify these different mechanisms.

The present findings in mice are expected to have important consequences in humans, since cognitive ability and intelligence, although known to be highly heritable traits [319], still have no clear genetic basis [320,321]. Such "missing heritability" of complex traits is classically postulated to be due to gene-gene and gene-environment interactions involving multiple, often rare gene variants that bring small effects [322]. Our results suggest that environmental factors encountered by parents also contribute to the heritability of cognitive abilities. Such a link is difficult to study in humans due to the complexity of the genome [318]. Animal models like ours therefore provide a valuable means to study the underlying mechanisms, and gain novel insight with potential implication for the clinic.

8.2.5 Supplementary Figures

Figure S1 (Bohacek et al.)

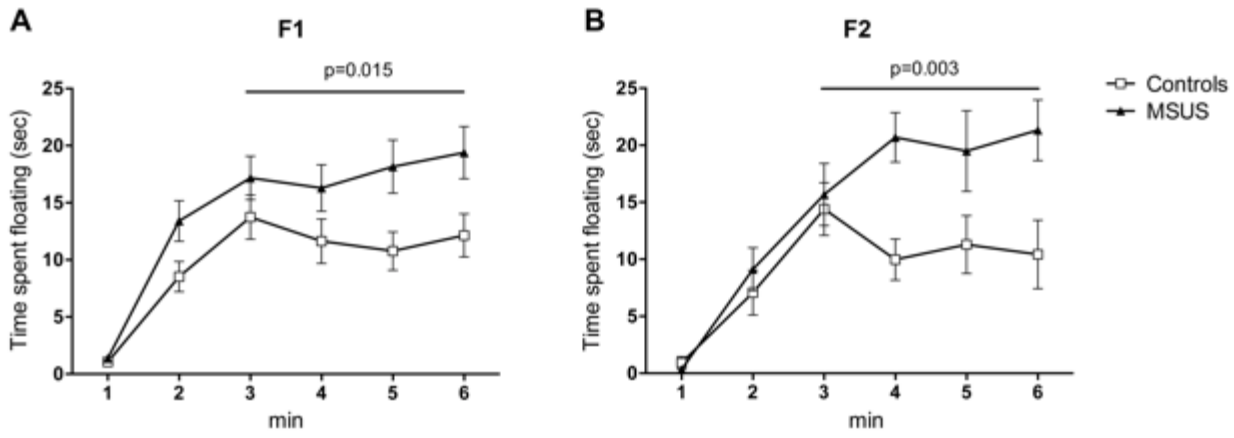

Figure S1. MSUS fathers and their offspring express depressive-like symptoms.
F1 MSUS males (A) and the female offspring (B) spend more time floating than controls. Data are mean ± s.e.m.

Figure S2 (Bohacek et al.)

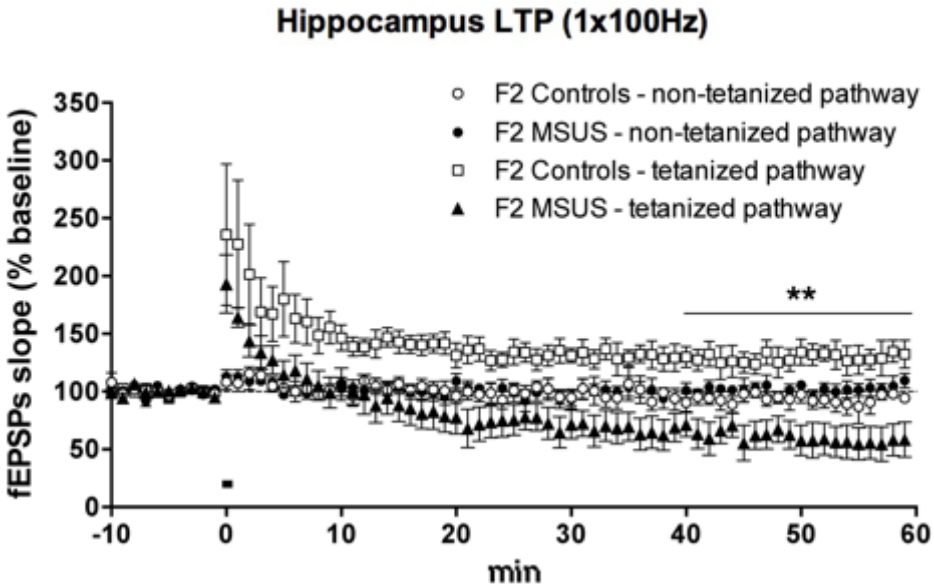

Figure S2. Two-pathway recordings in hippocampus of F2 mice.

Hippocampal LTP induced by 1x100Hz stimulation is impaired in F2 MSUS mice compared to controls (controls: 129.2 ± 10.3%; MSUS: 60.55 ± 12.9%; $t(10)=4.16$, $p=0.002$). Recordings from the second, non-stimulated pathway demonstrate a stable fEPSP response throughout recording (MSUS: n=6 mice; Controls: n=6 mice). Data are mean ± s.e.m. **=$p<0.01$ controls tetanized versus MSUS tetanized.

Figure S3 (Bohacek et al.)

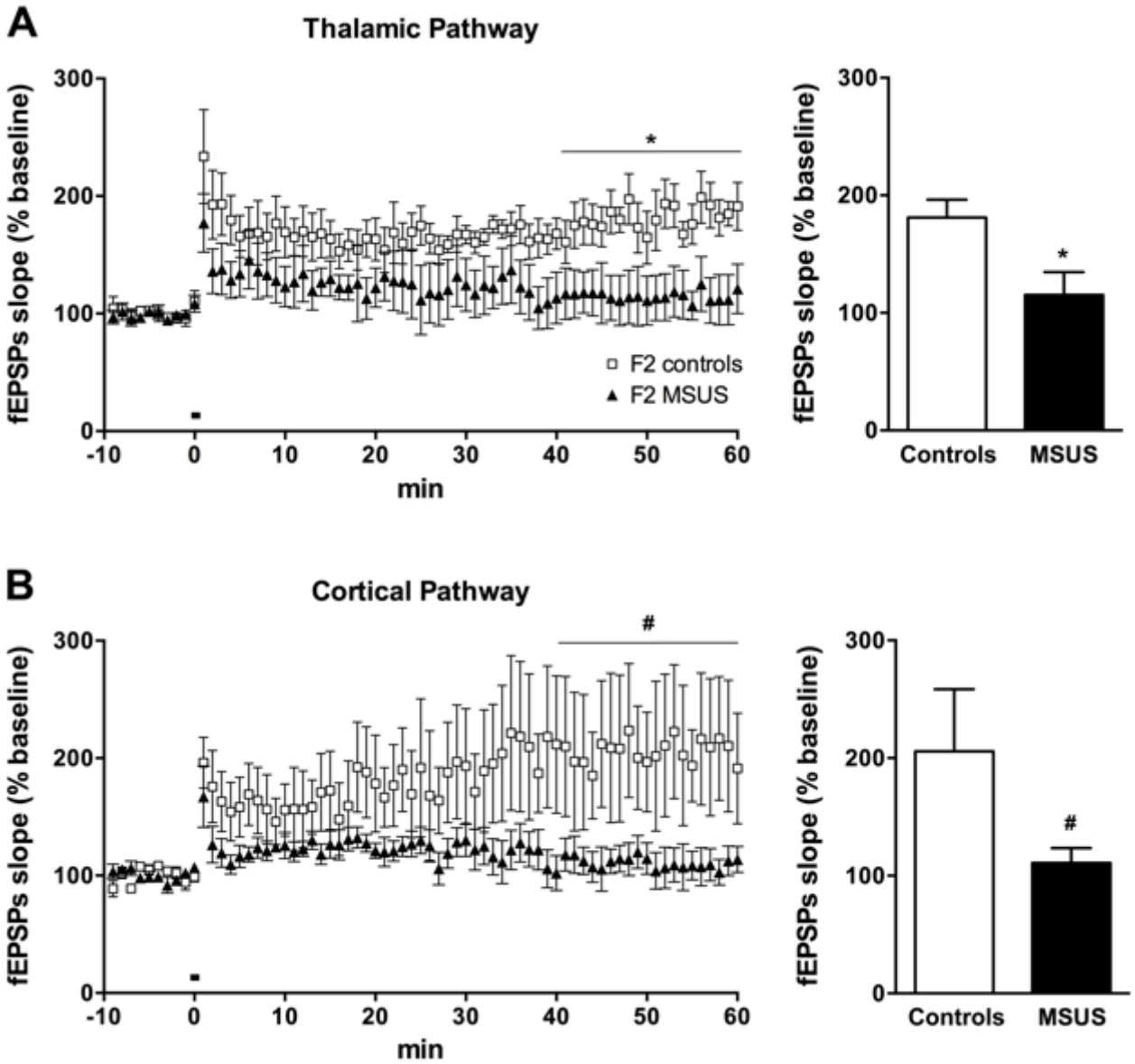

Figure S3. The offspring of MSUS males have impaired LTP in the amygdala.

(A) LTP induced at the thalamic pathway is impaired in F2 MSUS mice (n=7 mice) compared to controls (n=6 mice). (B) LTP induced at the cortical pathway is slightly impaired in F2 MSUS mice (n=7 mice) compared to controls (n=5 mice). Thick black horizontal bars indicate tetanus stimulation. Narrow horizontal bars indicate the last 20 min of recording used for statistical analyses. Bar graphs represent the fEPSP slope averaged across the last 20 min of recording. Data are mean ± s.e.m. *=p<0.05, #=p<0.07

8.2.6 Supplementary Tables

Supplementary Table 1 (Bohacek et al.)

MSUS VERSUS CONTROLS – DOWN-REGULATED PATHWAYS – ACUTE STRESS CONDITION

Rank	Gene Set Name	SIZE	ES	NES	NOM p-val	FDR q-val
1	**DARPP 32 events - Reactome Pathway**	**23**	**-0.6528**	**-2.356298**	**0.000000**	**0.008157**
2	Cytosolic tRNA aminoacylation – Reactome Pathway	24	-0.6278	-2.291540	0.000000	0.009718
3	**RAS activation upon Ca2+ influx through NMDA receptor – Reactome Pathway**	**14**	**-0.6892**	**-2.168596**	**0.000623**	**0.027765**
4	**GABA synthesis, release, reuptake and degradation – Reactome Pathway**	**19**	**-0.6157**	**-2.101296**	**0.000000**	**0.045921**
5	ARF1 Pathway - NCI / Nature Pathway	18	-0.6040	-2.048614	0.001346	0.068716
6	Glycolysis – Reactome	25	-0.5301	-1.984103	0.000000	0.110520
7	Circadian repression of expression by REV-ERBA – Reactome Pathway	21	-0.5537	-1.955585	0.000673	0.128240
8	**CREB phosphorylation through the activation of CaMKII – Reactome Pathway**	**13**	**-0.6316**	**-1.930020**	**0.003578**	**0.142792**
9	RORA activates circadian expression – Reactome Pathway	22	-0.5321	-1.899508	0.003353	0.169132
10	Attachment of GPI anchor to UPAR – Reactome Pathway	6	-0.8054	-1.889692	0.003676	0.167082
11	Beta-catenin phosphorylation cascade – Reactome Pathway	15	-0.5928	-1.885943	0.004531	0.156791
12	Trafficking and processing of endosomal TLR – Reactome Pathway	10	-0.6662	-1.877965	0.006150	0.153535
13	**AKT phosphorylates targets in the cytosol – Reactome Pathway**	**13**	**-0.6115**	**-1.866960**	**0.007304**	**0.155606**
14	N-Cadherin signaling events – NCI / Nature Pathway	29	-0.4845	-1.865257	0.001498	0.146645
15	Gluconeogenesis – Reactome Pathway	27	-0.4826	-1.829601	0.002187	0.183170
16	Formation of tubulin folding intermediates by CCT-TRIC – Reactome Pathway	15	-0.5618	-1.801884	0.008497	0.214504
17	Circadian clock – Reactome Pathway	47	-0.4123	-1.795617	0.001786	0.211789
18	Glucose metabolism – Reactome Pathway	56	-0.3991	-1.795491	0.000000	0.200196
19	Purine ribonucleoside monophosphate biosynthesis – Reactome Pathway	10	-0.6448	-1.794888	0.012479	0.190483
20	**PI3K-AKT activation – Reactome Pathway**	**36**	**-0.4370**	**-1.778563**	**0.002423**	**0.205005**
21	**Transmission across chemical synapses – Reactome Pathway**	**173**	**-0.3230**	**-1.768603**	**0.000000**	**0.209498**
22	Folding of actin by CCT-TRIC – Reactome Pathway	8	-0.6699	-1.760188	0.018395	0.213463
23	Prefoldin-mediated transfer of substrate to CCT-TRIC – Reactome Pathway	19	-0.4988	-1.738467	0.011392	0.238631
24	mRNA splicing – Reactome Pathway	94	-0.3450	-1.738288	0.000000	0.229013

25	mRNA splicing – major pathway – Reactome Pathway	94	-0.3450	-1.724625	0.002445	0.242496

MSUS VERSUS CONTROLS – DOWN-REGULATED PATHWAYS – BASELINE RESTING CONDITION

Rank	Gene Set Name	SIZE	ES	NES	NOM p-val	FDR q-val
1	Interaction between L1 and ankyrins – Reactome Pathway	24	-0.7557	-2.257383	0.000000	0.000000
2	L1CAM interactions – Reactome Pathway	89	-0.5084	-1.912260	0.000000	0.046290
3	CDO in myogenesis – Reactome Pathway	29	-0.5958	-1.846442	0.000128	0.068890
4	Myogenesis – Reactome Pathway	29	-0.5958	-1.859348	0.000514	0.074750
5	**Reelin signaling pathway – NCI / Nature Pathway**	**28**	**-0.5834**	**-1.799209**	**0.000896**	**0.113026**
6	**Unblocking of NMDA receptor – glutamate binding and activation – Reactome Pathway**	**15**	**-0.6548**	**-1.744675**	**0.002632**	**0.205848**
7	Axon guidance – Reactome Pathway	248	-0.4165	-1.722747	0.000000	0.206658
8	**Synaptic transmission – Reactome Pathway**	**150**	**-0.3990**	**-1.584540**	**0.001167**	**0.217212**
9	Other semaphorin interactions – Reactome Pathway	12	-0.6344	-1.585063	0.017888	0.221986
10	Stabilization and expansion of the E-cadherin adherens junction – NCI / Nature Pathways	62	-0.4440	-1.587115	0.005237	0.223520
11	ARF6 downstream pathway – NCI / Nature Pathway	82	-0.4627	-1.726816	0.000449	0.223844
12	E-Cadherin signaling in the nascent adherens junction – NCI / Nature Pathway	62	-0.4440	-1.587543	0.006009	0.228887
13	Dopamine neurotransmitter release cycle – Reactome Pathway	14	-0.6483	-1.705752	0.006517	0.228903
14	**CREB phosphorylation through the activation of CaMKII – Reactome Pathway**	**13**	**-0.6593**	**-1.696555**	**0.005491**	**0.231816**
15	Neurofascin interactions – Reactome Pathway	7	-0.7347	-1.587609	0.015335	0.235469
16	Interactions of the immunoglobulin superfamily – IgSF-member proteins – Reactome Pathway	33	-0.4908	-1.564844	0.015705	0.239350
17	**CREB phosphorylation through the activation of RAS – Reactome Pathway**	**23**	**-0.5227**	**-1.546832**	**0.022805**	**0.239404**
18	Serotonin neurotransmitter release cycle – Reactome Pathway	12	-0.6147	-1.548960	0.027837	0.239528
19	Activation of NMDA receptor upon glutamate binding and postsynaptic events – Reactome Pathway	33	-0.4926	-1.567301	0.013687	0.239533
20	**RAS activation upon Ca2+ influx through NMDA receptor – Reactome Pathway**	**15**	**-0.6274**	**-1.672524**	**0.007497**	**0.240002**
21	Alk2 signaling events – NCI / Nature Pathway	7	-0.7389	-1.588293	0.015715	0.240818
22	LKB1 signaling events – NCI / Nature Pathway	300	-0.3739	-1.561564	0.000101	0.241294
23	PDGFR-beta signaling pathway – NCI / Nature Pathway	38	-0.4768	-1.571220	0.011886	0.242319
24	cGMP effects – Reactome Pathway	19	-0.5501	-1.549473	0.024148	0.243350

25	Transport of inorganic cations/anions and aminoacids/oligopeptides – Reactome Pathway	88	-0.4174	-1.567619	0.002785	0.244797
26	EphrinB-EPHB pathway – NCI / Nature Pathway	40	-0.4810	-1.589420	0.009510	0.245537
27	CDC42_signaling events – NCI / Nature Pathway	199	-0.3814	-1.554728	0.000515	0.246470
28	Inactivation of CDC42 and RAC – Reactome Pathway	9	-0.7161	-1.663413	0.007069	0.247612
29	Attachment of GPI anchor to uPAR – Reactome Pathway	6	-0.7554	-1.549746	0.018757	0.247895
30	**Glutamate binding and activation of AMPA receptors and synaptic plasticity – Reactome Pathway**	**28**	**-0.5434**	**-1.676505**	**0.005809**	**0.247935**

MSUS VERSUS CONTROLS – UP-REGULATED PATHWAYS – BASELINE RESTING CONDITION

Rank	NAME	SIZE	ES	NES	NOM p-val	FDR q-val
1	Respiratory electron transport – Reactome Pathway	63	0.6370	2.946389	0.000000	0.000000
2	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins – Reactome Pathway	78	0.5879	2.792768	0.000000	0.000000
3	Influenza viral RNA transcription and replication – Reactome Pathway	92	0.4548	2.233631	0.000000	0.005975
4	mRNA splicing – minor pathway – Reactome Pathway	42	0.5081	2.155038	0.000553	0.014429
5	Viral mRNA synthesis – Reactome Pathway	14	0.6923	2.126668	0.001428	0.016374
6	Viral mRNA translation – Reactome Pathway	76	0.4426	2.100230	0.000000	0.018371
7	Eukaryotic translation termination – Reactome Pathway	76	0.4262	2.050785	0.000000	0.027008
8	Formation of a pool of free 40s subunits – Reactome Pathway	86	0.4137	2.019702	0.000000	0.033370
9	Peptide chain elongation – Reactome Pathway	76	0.4172	1.998227	0.000000	0.037169
10	Eukaryotic translation elongation – Reactome Pathway	79	0.4052	1.940087	0.000000	0.060635
11	Peptide ligand binding receptors – Reactome Pathway	153	0.3557	1.909695	0.000000	0.075031
12	Eukaryotic translation initiation – Reactome Pathway	103	0.3670	1.862322	0.000000	0.099083
13	CAP dependent translation initiation – Reactome Pathway	103	0.3670	1.869817	0.000000	0.100039
14	RNA polymerase I promoter opening – Reactome Pathway	22	0.5233	1.837691	0.004062	0.106987
15	RNA polymerase III transcription initiation from type 3 promoter – Reactome Pathway	22	0.5093	1.822229	0.001686	0.107281
16	GTP hydrolysis and joining of the 60s ribosomal subunit – Reactome Pathway	96	0.3615	1.808095	0.000000	0.108174
17	3'UTR mediated translational regulation – Reactome Pathway	96	0.3645	1.828253	0.000000	0.108592
18	Intrinsic pathway – Reactome Pathway	13	0.5939	1.800778	0.009240	0.109269
19	Regulation of gene expression in beta cells – Reactome Pathway	93	0.3686	1.810373	0.000000	0.112304
20	Pausing and recovery of TAT mediated HIV 1 elongation	29	0.4571	1.773288	0.004634	0.113769

	– Reactome Pathway					
21	mRNA capping – Reactome Pathway	28	0.4864	1.837865	0.003699	0.114385
22	Tachykinin receptors bind tachykinins – Reactome Pathway	5	0.8127	1.782317	0.008907	0.115320
23	Abortive elongation of HIV 1 transcript in the absence of TAT – Reactome Pathway	23	0.4967	1.786875	0.006650	0.116555
24	RNA Pol II CTD phosphorylation and interaction with CE – Reactome Pathway	26	0.4756	1.775288	0.006203	0.116822
25	Translation – Reactome Pathway	110	0.3463	1.763380	0.000000	0.118204
26	TAT mediated HIV 1 elongation arrest and recovery – Reactome Pathway	29	0.4571	1.745147	0.007480	0.130885
27	Influenza infection – Reactome Pathway	133	0.3291	1.719972	0.000000	0.152703
28	Glyoxylate metabolism – Reactome Pathway	4	0.8569	1.710628	0.008262	0.157669
29	Dual incision reaction in TC-NER – Reactome Pathway	28	0.4414	1.661481	0.010914	0.189147
30	Thyroxine biosynthesis – Reactome Pathway	5	0.7604	1.663282	0.028348	0.192601
31	Regulatory RNA pathways – Reactome Pathway	21	0.4712	1.653687	0.017666	0.193690
32	Influenza life cycle – Reactome Pathway	128	0.3225	1.675252	0.000000	0.196160
33	MicroRNA (miRNA) biogenesis – Reactome Pathway	21	0.4712	1.663841	0.012955	0.198098
34	Formation of transcription-coupled NER (TC-NER) repair complex – Reactome Pathway	28	0.4414	1.666733	0.011829	0.200764
35	RNA polymerase III chain elongation – Reactome Pathway	12	0.5554	1.629703	0.037248	0.202798
36	HIV 1 elongation arrest and recovery – Reactome Pathway	30	0.4200	1.630219	0.011854	0.207538
37	RNA polymerase III transcription initiation – Reactome Pathway	28	0.4266	1.620798	0.010658	0.209204
38	Pausing and recovery of HIV 1 elongation – Reactome Pathway	30	0.4200	1.630645	0.013315	0.212688
39	Elongation arrest and recovery – Reactome Pathway	30	0.4200	1.634678	0.017729	0.213119
40	Pausing and recovery of elongation – Reactome Pathway	30	0.4200	1.613256	0.009307	0.214012
41	Regulation of beta-cell development – Reactome Pathway	105	0.3159	1.591330	0.000000	0.222677
42	Formation of HIV 1 elongation complex containing HIV 1 TAT – Reactome Pathway	40	0.3808	1.583066	0.010352	0.223899
43	Regulation of insulin-like growth factor (IGF) activity by insulin-like growth factor binding proteins (IGFBPS) – Reactome Pathway	16	0.4893	1.592934	0.031826	0.225693
44	Pyrimidine salvage reactions – Reactome Pathway	7	0.6452	1.585097	0.050695	0.226014
45	HIV 1 transcription elongation – Reactome Pathway	40	0.3808	1.594623	0.008830	0.228727
46	TAT mediated elongation of the HIV 1 transcript – Reactome Pathway	40	0.3808	1.598296	0.010281	0.229238
47	Integration of provirus – Reactome Pathway	7	0.6318	1.561780	0.054626	0.243970
48	ID pathway – Cancer Cell Map	10	0.5669	1.558317	0.054372	0.244015
49	Phosphorylation of EMI1 – Reactome Pathway	5	0.7184	1.561835	0.056467	0.249080

Supplementary Table 2 (Bohacek et al.)

RT-qPCR primers

GENE	Forward Primer	Reverse Primer
Prkcc	TGTGGCCATCTGCAAAGGGTTC	ACCTCTCCCAATCGATCCAACG
Grin1	TGTGTCCCTGTCCATACTCAAGTC	CCGAACCCATGTCTTATCCAGGTC
Grm1	AGTGCCTTCACCACCTCTGATG	ATTCTGGCTGCCTCTTCTTGGC
Camk2a	TGAGGACGAAGACACCAAAGTGC	CTTCGATCAGCTGCTCTGTCAC
Gria3	ACGGGCAGAGTCCAAACGCA	CGTGTCATGCCCGACACCAA
Tubd1	TCTCTTGCTAACTTGGTGGTCCTC	GCTGGGTCTTTAAATCCCTCTACG
Hprt	GTTGGGCTTACCTCACTGCTTTC	CCTGGTTCATCATCGCTAATCACG
Actb	TGTTACCAACTGGGACGACA	TTTGAGACCTTCAACACCCC
Gapdh	CAGCAATGCATCCTGCACC	TGGACTGTGGTCATGAGCCC

8.2.7 Materials and Methods

Animals

C57Bl/6J females and males (2.5 months) were obtained from Janvier (France) and maintained in a temperature- and humidity-controlled facility on a 12-hour reversed light–dark cycle with food and water *ad libitum.* All procedures were carried out in accordance to Swiss cantonal regulations for animal experimentation.

Unpredictable maternal separation combined with maternal stress (MSUS) and breeding paradigm

MSUS was conducted as previously described [264,272]. Briefly, first generation (F1) pups were separated from their mothers for 3 hrs per day at unpredictable times from postnatal day 1 to 14. During separation, mothers were randomly exposed to restraint stress (20 min) or forced swim stress (5 min). At postnatal day 21, pups were weaned and placed in standard cages (3-5 mice per cage, each from a different litter to avoid litter effects). When adult, males subjected to MSUS and controls (F1) were bred to naïve wild-type females to produce an F2 generation. At least 10 males were used for breeding in each group. F2 mice were reared in normal conditions not involving any stress (3-5 mice per cage, each from a different litter to avoid litter effects). To generate F3 offspring, F2 males were paired to naïve females as described for the F2 breeding.

Cross-fostering design

When F2 mice were born, 3 litters per group were pseudo-randomly (matched for litter size and birthdate) selected for cross-fostering. These litters where removed from their mother within 48 hrs after birth and immediately placed with a foster-mother from the opposite experimental group (MSUS vs. control). Donor and recipient dams were removed from their cage and placed in separate, clean holding cages. The litter to be fostered was picked up and placed in the recipient's homecage and gently mixed with the dirty bedding to transfer the recipient's scent [323]. Then the recipient dam was returned to the cage and the pups were henceforth raised under normal facility and rearing conditions. Maternal care was determined by daily visual inspection.

Gene expression analyses

F2 female mice (MSUS and controls) were sacrificed by cervical dislocation either 45 min (acute stress condition) or 2 weeks (baseline resting condition) after exposure to forced swim stress (6 min, 18°C water) (conducted as two independent experiments in independent breeding cohorts of mice). The brain was removed and the hippocampus rapidly dissected on ice and stored at -80°C until further processing. RNA was extracted with RNeasy spin columns (Qiagen) and amplified using the Ovation RNA amplification kit V2 (Nugen Technologies). RNA was labeled, hybridized and analyzed using NimbleGen Mouse Gene Expression 12x135K Arrays according to manufacturer's recommendations. Independent experiments were conducted for baseline resting condition and for acute stress condition. Each RNA sample (6 samples per group for baseline resting condition, and 8 samples per group for acute stress condition) was hybridized to two different NimbleGen slides, providing a technical replicate for each measurement.

Quality control and statistical analysis of microarray data

Principal Component Analysis (PCA) demonstrated high reproducibility between technical replicates in both experiments and identified one low quality replicate in baseline resting conditions that was removed from further analyses (the second technical replicate was not affected). After scanning, data were processed with RMA (Robust Multichip Average) [324] according to NimbleGen's recommendation. For analysis of differential expression, the expression matrix was log2 transformed and imported into Partek Genomics Studio (Partek Inc, Missouri, USA) and Limma [325]. To identify probes for genes that are differentially

expressed in both groups, a linear model was run. Multiple testing corrected p-values (FDR method) were calculated for the contrast between groups in both experiments.

Gene set enrichment analyses (GSEA)

GSEA algorithm implemented in the GSEA tool from Broad Institute [326] was used to detect coordinated changes in gene expression in biological networks. This gene-set based approach is particularly powerful for datasets where overall expression analysis proves insensitive [244]. T-statistic values (on a gene level) from the ANOVA model described above were used as a measure of change for all genes whose mouse gene ID could be mapped to a human ortholog, based on the Roche genome annotation infrastructure. As a gene set library, we used the Pathway Commons [327] collection, limiting down to the pathways that contain between 5 to 500 genes. Significantly regulated pathways were identified within GSEA by performing random permutations in the gene space, the number of permutations set to 10000. The lists of significantly up- or down-regulated pathways were sorted by FDR corrected p-values, set to the recommended 25% threshold [326].

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

RT-qPCR was performed using SYBR green (Roche) on a Light-Cycler II 480 (Roche) according to manufacturer's recommendations and normalized against Tubulin delta 1 (Tubd1). Cycling conditions were 5 min at 95°C, then 45 cycles with denaturation (10 sec at 95°C), annealing (10 sec at 60°C), and elongation

(10 sec at 72°C). Primers were designed using Primer3Plus [328] or Quantprime [329] (see Supplementary Table 2) and tested for quality and specificity by melt-curve analysis, gel electrophoresis and appropriate negative controls.

Bisulfite pyrosequencing

Genomic DNA was extracted and purified using DNeasy Blood and Tissue Kit (Qiagen), and bisulfite treated (EZ DNA Methylation Gold Kit, Zymo Research) according to manufacturer's recommendations. PCR and pyrosequencing primers were designed using Pyromark Assay Design 2.0 (Qiagen). Amplicons containing the *Prkcc* promoter region were generated using a standard PCR protocol, an unmodified forward primer (AAGATGATTGATTGATTGGGAGAA), and a biotin-labeled reverse primer (ACACCTAACCATACACAACACAC). Subsequent pyrosequencing on the PCR amplicon was performed using a PyroMark Q24 Advanced pyrosequencer and appropriate reagents (Pyromark Advanced CpG Reagents, Qiagen) following manufacturer's recommendations. For high-resolution sequencing, the following two sequencing primers were used (sequencing primer1: AAGGGGGTGGATAAG; sequencing primer2: GGGGGTTTTAAATTGAAAT). Average methylation levels of CpG sites were quantified using PyroMark Q24 2.0 software (Qiagen).

Electrophysiology

Adult F2 female and male mice were used for electrophysiological experiments. Mice were anesthetized with isoflurane and rapidly decapitated. Heads were immediately immersed in ice-cold freshly prepared artificial CSF (aCSF: 119 mM

NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.3 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 11 mM $C_6H_{12}O_6$) before brain extraction. aCSF was gassed with 95% O_2 and 5% CO_2. The hippocampus was isolated and sagittal slices (400 µm) were prepared with a LEICA VT 1000S vibratome. Slices were allowed to recover at 34°C for at least one hour, then placed in an interface chamber at room temperature and perfused with aCSF. Extracellular fEPSPs were recorded with a glass microelectrode (2-5 MΩ filled with aCSF) positioned in the stratum radiatum of area CA1. A concentric bipolar stimulating electrode was used to elicit fEPSPs by stimulation of Schaffer collateral fibers. Basal synaptic transmission was assessed by stimulating Schaffer collaterals with increasing stimulation intensity (I/O curves). Stimulation intensity was adjusted to elicit fEPSP amplitudes that were around 50% of maximum size. LTP was induced by applying either a single 1-sec train (100 Hz at test strength) or three 1-sec trains (100 Hz, test strength) 20 sec apart. LTD was induced with 1200 pulses at 2Hz for 10 minutes, repeated three times at 10 min intervals [330]. Paired-pulse facilitation (PPF) was induced with two stimulations delivered at an intertrial interval (ITI) of 50, 100, 150, 200, 300, or 400 ms, and was determined as the ratio of fEPSP slope evoked by the second pulse to fEPSP slope evoked by the first pulse (EPSP2/EPSP1). For amygdala recordings, horizontal slices were prepared and the recording electrode was placed in the lateral nucleus of the amygdala. To stimulate fibers originating from the thalamus (thalamic pathway), one stimulation electrode was placed close to the internal capsule, while another stimulation electrode was placed externally to the capsule to stimulate fibers from the auditory cortex

(cortical pathway) [184]. LTP was induced with one 1-sec train at 100 Hz (at test strength). Signals were amplified with an Axopatch 200B amplifier (Molecular Devices, Union City, CA) digitized by a *Digidata 1322A interface* (Axon Instruments, Molecular Devices, US) and sampled at 10 kHz. Recordings were acquired using Labview (National Instruments) and analyzed with Clampfit (Molecular Devices). Experimenters were blind to treatment for all experiments. One or more slices from each mouse were used and data were averaged, so that animals and not slices were considered biological replicates.

Behavioral testing

Behavior testing was conducted in adult (3-8 month-old) male mice to avoid confounding effects of estrous cycle. Testing was carried out under dim red light and animals were monitored by direct observation and/or videotracking. Each animal was tested on a maximum of three tasks, 1 to 2 weeks apart, starting with the least aversive task. An experimenter blind to the treatment conditions conducted all tests and analyses.

Forced swim test. Mice were placed in a plastic cylinder (18 cm high, 13 cm diameter) filled with 18 ± 1°C water up to 12 cm height for 6 min. Time floating was manually scored and the last 4 min were used to compare performance in the different groups [331].

Novel object recognition. Mice were habituated to an arena (grey plastic box, 25x25x20 cm) in a dimly lit room for 10 min on 3 consecutive days. They were then allowed to explore three identical unfamiliar objects in the arena for 10 min. After 2.5 hrs, they were placed back in the arena but one of the objects was

replaced with a novel object. Using the same animals, this test was then repeated using different objects and a 24-hr delay to test long-term memory. The arena floor was lit with infrared light and animals were tracked with an infrared camera and tracking system (Viewpoint, France). Time spent exploring each object was recorded normally.

Contextual fear conditioning. Mice were exposed to a novel context for 3 min in an automated fear conditioning system (TSE, Germany), then received two 1 sec 0.6 mA foot-shocks 1 min apart. Movement was detected by infrared beams in the testing chamber (TSE, Germany). Freezing, defined as the absence of any detectable movement for >1 sec, was measured for 4 min in the same context immediately before and 24 hrs after fear conditioning and served as an indicator of memory.

Statistical analyses

For RT-qPCR and pyrosequencing analyses, independent samples t-tests were used to compare both groups. For electrophysiology, independent samples t-tests were used to compare the fEPSP slope over the last 20 min of LTP/LTD recordings between groups. For object recognition memory, ANOVA was used with object as within-subjects factor. Significant main effects were further analyzed using paired-samples t-tests to compare the average time spent exploring the familiar objects and time spent exploring the novel object. Fear conditioning data were analyzed using independent samples t-tests. Values were considered outliers if they deviated > 2 SDs from the group mean, and this outlier exclusion criterion was pre-established.

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