UNIVERSITY OF CALIFORNIA SAN DIEGO

A recipe for memory: Identifying a fear engram

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in

Neurosciences

by

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The Dissertation of Leonardo Minete Cardozo is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

2019

Dedication

To Iuri Ventura,

my joy

Fundamental é mesmo o amor, é impossível ser feliz sozinho.

Love is genuinely fundamental, it is impossible to be happy alone.

or

The fundamental loneliness goes whenever two can dream a dream together.

Tom Jobim

Wave, 1967

Epigraph

Has it ever struck you, Connie, that life is all memory, except for the one present moment that goes by you so quick you hardly catch it going?

Tennessee Williams

The Milk Train Doesn't Stop Here Anymore, 1963

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Acknowledgments

Before the acknowledgements, I would like to say that the pursue of a PhD thought me about the impact of decisions in life and what really matters. It thought me it is a huge mistake to put career in front of everything else, especially if that means a commitment of five plus years. It took a toll on my health, with chronic anxiety and back pain, which may never go away completely. I am sure I was only able to get through my PhD because of the support of so many especial people in my life, whom I acknowledge here.

It feels odd to see my name as the only one in the title page of this dissertation. The truth is that many people helped me, devoting countless hours of their lives, so that I could get to the end of my PhD and write this piece. By acknowledging them here, I hope to, at least in part, recognize their fundamental support to me and to this work.

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ABSTRACT OF THE DISSERTATION

A recipe for memory: Identifying a fear engram

by

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The fundamental question that motivates this thesis is the elusive physical basis of memory. Despite substantial research advances over the last decades, the cellular and molecular mechanisms that allow the brain to create, store and recall memories remain largely unknown, mainly due to the lack of techniques to test the causal role of learning-induced modifications *in vivo*. We propose an approach to identify an engram - or memory trace - for contextual fear conditioning, a paradigm that involves associating a neutral context (conditioned stimulus, CS) with mild foot shocks (unconditioned stimulus, US), producing an aversive memory to the

context. Our approach for identifying an engram is based on three criteria: (1) an observable learning-induced change that (2) once disrupted impairs memory; and (3) if artificially induced can produce a memory independent of associative training. Our hypothesis is that synaptic potentiation in neurons of the basolateral amygdala, a known fear center that responds to both CS and US stimuli, represents an engram for context conditioning. We found that conditioning produced potentiation of excitatory synapses that lasted for at least 7 days specifically onto basolateral amygdala neurons activated during learning (1). We developed a method to disrupt memories by reversing synaptic potentiation specifically in learning-activated neurons using a mutated form of CaMKIIα that induces synaptic depression, demonstrating a causal role between synaptic plasticity and memory recall (2). Lastly, we generated an artificial fear association, without associative Pavlovian conditioning, by high frequency optogenetic stimulation of the CS and US-activated ensembles or biochemical induction of synaptic potentiation in US-responsive neurons, demonstrating the sufficiency of synaptic potentiation of amygdala neurons in producing context fear memories (3). These results suggest that a necessary and sufficient step in forming context fear associations is plasticity of CS inputs onto US-responsive basolateral amygdala neurons and that context discrimination is determined by the CS-specific amygdala inputs activated during retrieval. Synaptic plasticity found elsewhere in the hippocampus and other cortical regions during context conditioning might be more involved in context encoding, modulating the response to the aversive memory stored in the basolateral amygdala.

Chapter 1: Introduction

Every act of memory is to some degree an act of imagination.

Gerald Edelman and Giulio Tononi
A Universe of Consciousness, 2000

The fundamental problem that motivates this thesis is the unknown physical basis of memory. Despite substantial research advances over the last decades, the cellular and molecular mechanisms that allow the brain to create, store and recall memories remain elusive. As stated by Kandel and colleagues (Kandel et al., 2014), "memory is the glue that holds our mental lives together". Memory is the basis for most, if not all, cognitive processes. Without memory, there is no language, no history, no abstract thought. Therefore, true comprehension of high cognition relies on unraveling the mechanisms behind learning, memory storage and recall. Due to our poor understanding of these processes, several interesting questions remain unanswered: Is a given memory stored in a small subset of neurons, or distributed over a large network of neurons? Is there a "grandmother" neuron (Quiroga et al., 2008, 2005)? Is there a set of molecular components that lasts for as long as a given memory lasts? Do all types of long-term memories rely on the same molecular mechanisms [e.g. declarative vs procedural memory (Squire, 2004)]? How do forgetting and memory extinction work? Is it possible to develop therapies targeting the molecular mechanisms of memory to enhance encoding and/or forgetting? The goal of this thesis is to advance our knowledge of the cellular mechanisms of memory, shining light on potential answers to some of these questions.

In this chapter, I give some historical perspective on the search for the engram or memory trace, exploring why it represents the fundamental problem in learning and memory, and how the search benefits from recent technical advances in neuroscience. I discuss the

controversy behind the different criteria proposed for identifying an engram, and I argue that there is a set of criteria that properly define the conditions necessary for identification of a memory trace. Moreover, I briefly review the potential mechanisms responsible for memory storage and discuss the studies that provided the best evidence to date on identifying the engram. Finally, I summarize the relevant literature on Contextual Fear Conditioning, which was the behavioral paradigm used in this thesis, and state the main hypothesis that was tested in my doctoral studies.

1.1. The search for the engram: a historical perspective

For the purposes of this thesis, the engram is defined as the learning-induced physical modifications that underlie memory. Therefore, the search for the engram represents the fundamental question regarding the elusive physical basis of memory. For clarification, in this thesis, the terms "engram" and "memory trace" have the same meaning and will be used interchangeably.

I would like to stress the importance of two points in the definition of the engram, or memory trace:

- The engram is a physical modification (e.g. change in synaptic strength, or excitability), not a representation, not a cell or a group of cells (unless the cells were produced as the result of a learning process).
- These learning-induced physical modifications underlie memory, so the mere correlation between a physical modification with learning doesn't address their contribution to memory storage and recall. Numerous experience dependent

> modifications in the neural circuit may be simply due to homeostatic regulation and not directly participate in memory recall [although learning per se might induce homeostatic plasticity (Turrigiano, 1999)].

Another important point is that the engram does not represent what is necessary or sufficient for memory recall. A visual memory might depend on retinal neurons to provide sensory cues to be triggered; a motor memory requires muscles and bones to be recalled, but arguably none of these body parts stores the engram.

The term "engram" was introduced by Richard Semon in 1904, and was defined as "the enduring though primarily latent modification in the irritable substance produced by a stimulus" (Semon, 1923, 1921). His framework emphasized the relevance of retrieval, as memory emerges from an interaction between stored information and retrieval cues (Josselyn et al., 2017; Schacter, 2012; Schacter et al., 1978). Semon's work remained restricted to the theoretical aspects of memory given the technical limitations of the time: "To follow this into the molecular field seems to me… a hopeless undertaking at the present stage of our knowledge; and for my part I renounce the task" (Semon, 1923, p 154). Despite influencing Bertrand Russel in his book *Analysis of Mind* (Russell, 1921), personal and professional drawbacks, and Lamarckian ideas seem to have contributed to his ideas being largely ignored by the scientific community for decades until Schacter and colleagues revived them (Josselyn et al., 2017; Schacter, 2012; Schacter et al., 1978).

Although coined by Semon, the term "engram" was popularized by the American psychologist Karl Lashley. In his book *In the Search of the Engram* (Lashley, 1950), he synthetized over thirty years of work. Lashley pioneered the systematic use of thermocautery

brain lesions to study memory in experimental animals. Although considered a crude technique by today's standards (causing irreversible damage, including fibers of passage), it was one of the best available tools at the time to probe the function of a given brain region. Lashley's approach consisted of removing different portions of the cortex after training rats to navigate in a complex (Hebb-Williams) maze. His logic was that a memory deficit associated with a given lesion would suggest that the engram was located in the ablated cortex. After many iterations of this experiment, he concluded that the size, but not the location, of the lesion correlated with the magnitude of memory impairment, suggesting that the engram was not localized in a given brain region but distributed throughout the cortex. Given what we know today, I offer two possible explanations for Lashley results: (1) An important caveat of Lashley's work, due to its technical limitations, is that he was not probing for the engram - the physical modifications that underlie memory - instead he was looking for the neural circuit involved in triggering the memory of the maze and navigating it. Given the complexity of the task, most of cortex might well contribute to the animal's performance even though it did not undergo cellular modifications necessary for the memory (e.g. cortical regions responsible for sensory processing might be necessary for task execution even if not part of the engram). (2) As pointed out by Peter Milner, Lashley's lesions often damaged structures located beyond the neocortex, including the hippocampus (Milner, 1999). Given the current knowledge regarding the role of the hippocampus in spatial memories and rodent navigation, it might well explain why larger lesions, which more likely affected the hippocampus and subcortical structures such as the thalamus, led to more pronounced impairments in performance.

Interestingly, a few years after Lashley's publication on the elusive nature of the engram and the suggestion that the memory was distributed throughout the cortex and that memory

storage could be redundant, Brenda Milner and colleagues started reporting on influential work describing the profound effects of temporal lobe removal on long-term memories (Penfield and Milner, 1958; Scoville and Milner, 1957). The authors evaluated 10 patients with different lesions that varied in size and location and concluded that the hippocampal formation and the surrounding tissue was critical for the formation of new memories and for the recall of recent ones, contradicting Lashley's findings. Furthermore, Milner's work demonstrated that a specific type of memory was affected, known as declarative or episodic memories, while other types of memory including language and skills, known as non-declarative memories, were spared, suggesting that different types of memory rely on different brain regions (Squire, 2004).

Another set of important experiments in the search for an engram was carried out decades later by the group of Richard Thompson on eye-blink conditioning in rabbits. This paradigm consists of pairing an auditory tone, the conditioned stimulus (CS), with an aversive air puff to the cornea, the unconditioned stimulus (US), which reliably elicits eye closure. Naïve rabbits do not blink when the auditory tone (CS) is played but, with training, rabbits learn to blink to the CS, anticipating US delivery (Freeman and Steinmetz, 2011).

Thompson and colleagues defined two criteria that must be met to conclude that a given brain region contains an engram for eye-blink conditioning. First, learning-induced changes that correlate with the learned behavior must be observed in the candidate region. Second, ablation of the region "must both prevent acquisition and abolish retention of the learned behavioral response without impairing the response itself, that is, as a reflex response" (Steinmetz et al., 1992). One of their targets was the hippocampal region, which displayed an increased firing rate after learning (Berger et al., 1980; Berger and Thompson, 1978). However, lesioning the hippocampus failed to impair training or performance of learned behavior

(Solomon et al., 1986). Nevertheless, a series of experiments showed that the interpositus nucleus of the cerebellum satisfied both criteria (McCormick and Thompson, 1984; Steinmetz et al., 1992), even when physical lesions were substituted for reversible muscimol inactivation of neural activity(Krupa et al., 1993).

The series of experiments presented by Thompson and colleagues along with knowledge of the circuitry involved in eye blink conditioning presents a compelling case for the localization of an engram in the cerebellum. However, I would like to point out a caveat to Thompson's criteria for engram identification (Steinmetz et al., 1992): regions that are part of the CS pathway alone might satisfy the criteria; even sensory neurons in the ear that are required for processing the auditory tone might display some plasticity that correlates with the learned behavior without contributing to the CS-US association. Given the role of the cerebellum in sensory processing, it would be important to evaluate the impact of lesioning/inactivating the interpositus nucleus in auditory perception (Bower, 1997). Also, while Thompson observed learning-induced modifications in neuronal activity, they did not directly manipulate them to test causality (which is understandable considering the technical limitations of the time but should be highlighted given the current standards). Lastly, it is impressive that the interpositus nucleus of the cerebellum satisfied their second criterion, but in principle, the engram for the CS-US association might well be located in the same brain region that is required for the unconditioned response, i.e. ablation of the region would affect performance even without training. Nevertheless, Thompson and colleagues provided the most convincing set of experiments of the 20th century to link a brain region to the engram of a learned behavior in mammals (Josselyn et al., 2017).

Thompson's approach and set of criteria have had a significant impact on the current efforts to finding engrams. With the advent of techniques such as transgenic mouse lines for labeling active neurons, viral vectors and optogenetics, researchers now can refine the search for the engram to a group of neurons, or neuronal ensembles, within a given brain region instead of looking at the role of an entire region (Josselyn et al., 2015; Mayford, 2014; Tonegawa et al., 2015). There are two main strategies that haven been used to tag neuronal ensembles associated with a given behavior:

- The use of transgenic mice based on immediate early genes (IEGs, such as c-fos, and arc), which are genes that are activated rapidly and transiently in response to a variety of cell stimuli, including neuronal activity (Mayford, 2014; Reijmers and Mayford, 2009; Reijmers et al., 2007; Tonegawa et al., 2015).
- The overexpression of cAMP-response element-binding protein (CREB) (Han et al., 2009, 2007; Josselyn et al., 2015; Park et al., 2016; Zhou et al., 2009) or other cellular component that increase neuronal excitability in a group of neurons (Yiu et al., 2014), which bias neuronal allocation to those neurons during a learning episode.

Such tagging strategies have been used in combination with techniques such as viral vectors or other mouse lines that induce the expression of genetic reporters to observe plasticity occurred during/after training (Choi et al., 2018; Matsuo et al., 2008). Alternatively, they may be combined with modulators of neuronal activity (such as inhibitory or excitatory opsins) to ablate neurons (Han et al., 2009) or inhibit neuronal activity, impairing memory recall (Denny et al., 2014; Gore et al., 2015; Tanaka et al., 2014), or to artificially activate neurons, inducing

apparent memory recall (Cowansage et al., 2014; Gore et al., 2015; Liu et al., 2012; Ramirez et al., 2013). However, this kind of approach has two important limitations:

- The observation of synaptic plasticity with learning only establishes correlation, not ruling out homeostatic change or modulatory role, which may influence memory recall but is not part of the engram.
- Ablating/inhibiting neuronal ensembles, or activating them, provide valuable information about the circuit involved in memory recall, but does not automatically indicate where the engram is located. Essential parts of a memory circuit may simply be involved with the expression of a given memory: for instance, neurons in the retina might be necessary and sufficient to recall a memory that relies on visual cues, even if they do not store any physical trace of it (Mayford, 2014).

Recently, several prominent researchers in the memory field have suggested an alternate use of the term engram. Despite defining the term "engram" as the memory trace, or the physical substrate of memory, with a very similar (if not equal) definition to the one used in this thesis, the term has at the same time been used as a synonym to "memory circuit", i.e. the neuronal circuit involved in retrieving the memory from partial cues (Eichenbaum, 2016; Josselyn et al., 2015; Tonegawa et al., 2018, 2015). These studies put no emphasis in investigating the role of learning-induces changes, which are the core of the engram. Instead, the mere observation of synaptic plasticity in the neuronal ensembles being investigated seemed to suffice. Therefore, this suggests that the definition of the term "engram" might be evolving, from the more molecular "physical substrate of memory" to the more cellular "memory circuit", and new terms might be coined in the future to refer to the memory trace.

Dissecting an engram requires specific manipulation of learning-induced modifications in the neural network. Next, I will present and discuss the set of criteria to identify an engram that best captures the essence of the memory trace. Later, I will discuss the cellular mechanisms and the behavioral evidence that best satisfies these criteria in mammals.

1.2. Criteria for identification of an engram

I propose the following criteria to determine if a candidate form of plasticity in a neuronal ensemble is (part of) the engram (or memory trace), derived from a similar set of criteria proposed previously (Martin et al., 2000; Mayford, 2014):

1. Detectability (Correlation): To identify a learning-induced form of plasticity (cellular change) in a specific subset of neurons, which are known to be part of the circuit and receive relevant inputs representing the components of the learned association being studied. This is the criterion for which the most evidence have been accumulated to date.

2. Blocking/Reversal (Causality or Necessity): To block/impair the candidate plasticity mechanism in the neuronal ensemble and inhibit/reverse a learned association. Some studies partially address this criterion using techniques that interfere with much more than just the candidate plasticity mechanism (e.g. ablating neurons). More restricted manipulations of candidate plasticity mechanisms have been recently carried out and will be discussed below.

3. Mimicry (Sufficiency): To mimic the induction of the candidate plasticity mechanism in the identified subset of neurons or synapses and produce a memory

independent of behavioral training. Evidence towards this criterion need to be carefully investigated, as interdependence between events of plasticity might exist, and a required form of learning-induced plasticity (i.e. part of the engram) might not be sufficient to induce memory recall. The evidence for this criterion is currently scarce.

This set of criteria covers the most important points associated with identifying neurons that store the physical substrate of memory. Over the years, with the advance of knowledge and techniques, the concept of "neuronal ensembles" as defined today might get obsolete. Therefore, these criteria might require revision and adaptations, but their logical principles should stand the test of time.

1.3. Mechanisms for memory storage

In 1894, Santiago Ramón y Cajal proposed that memory is stored in the growth of new connections (Ramon y Cajal Santiago, 1894). In the first half of the 20th century, this idea competed with the hypothesis from Karl Lashley and a number of Gestalt psychologists, which proposed that memory is stored in the "bioelectric field" generated by the aggregate activity of many neurons, rather than in a single cell or synapse (Kandel and Spencer, 1968), similar to the current proposed mechanism for working memory, or short-term memory (Serences, 2016). Donald Hebb, who conducted his PhD with Lashley (Josselyn et al., 2017), elaborated on Cajal's prescient idea, and in his influential book *The organization of behavior: a neuropsychological theory* proposed that the synaptic strengthening between groups of neurons that are active during an event provides the basis for long-term memory (Hebb, 1949). Hebb's theory of cell assemblies, or neuronal ensembles, and the hypotheses that memory is stored in

synaptic connections throughout the circuit, and that recall involves the recapitulation of the learning activated ensemble, still guides contemporary thought in memory research.

Experiments conducted by Eric Kandel and colleagues on the gill-withdrawal reflex of the Aplysia provided the first set of evidence that learning results from changes in the strength of synaptic connections (Castellucci et al., 1970; Kandel, 2001; Kupfermann et al., 1970). However, the description of a Hebbian mechanism that would support long-term memory storage in synaptic connections in mammals came only decades later, with the discovery of Long Term Potentiation (LTP) (Bliss and Lomo, 1973). Over the decades, the mechanistic principles of LTP have been dissected, especially in the CA1 region of the hippocampus (Nicoll, 2017). The properties identified for the N-methyl-D-aspartate receptor (NMDAR)-dependent LTP, including input specificity, cooperativity and associativity, makes it a strong candidate mechanism of memory storage, following Hebbian rules (Malenka, 2003).

NMDAR are voltage and ligand-gated channels, which means that they are not activated by their ligands (glutamate, and glycine or D-Serine) at hyperpolarized potentials, due to the binding of the ion Mg^{2+}, which blocks the channel (Mayer et al., 1984; Nowak et al., 1984). When the neuron is depolarized, releasing the Mg^{2+} ion block, even weak synaptic activity is sufficient to activate NMDARs, allowing the influx of calcium, which initiates a cascade of Ca^{2+}-dependent plasticity events involving the Ca2+/calmodulin-dependent protein kinase II (CaMKIIα), and culminating in synaptic strengthening (Lisman et al., 2012; Nicoll, 2017), through the insertion of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPARs) in the dendritic spine (Bliss et al., 2003; Rumpel et al., 2005). It has been show in flies that the Mg^{2+} block in NMDARs is crucial for long-term memory formation (Miyashita et al., 2012).

Among the LTP properties, associativity and input specificity might be the ones that are more attractive to support models of memory storage. Input specificity is the fact that LTP is confined to specific spines, although modulated by dendritic branches (Branco and Häusser, 2010; Govindarajan et al., 2006) while associativity refers to the fact that weak stimuli, incapable of inducing LTP by themselves, can be potentiated through pairing with a strong stimulus, which is able to depolarize the membrane and release the Mg^{2+} block in NMDARs (Malenka, 2003). Such a property makes LTP an attractive mechanism for associating two pieces of information being conveyed by different sets of afferents that synapse on the same postsynaptic cell. Indeed, LTP has been recorded right after associative training (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Tsvetkov et al., 2002; Whitlock et al., 2006) providing correlational evidence that is involved in memory storage.

The biggest caveat with LTP serving as a mechanism of memory storage is its persistence; most LTP measurements *in vivo* show decay, returning to baseline within a few days or weeks (Abraham, 2003; Abraham et al., 1995). However, most measurements were conducted in the hippocampus, so it is possible that such a decay in LTP is related to the diminishing role of the hippocampus in memory storage over time due to systems consolidation (Frankland and Bontempi, 2005). It is unclear then if LTP lasts for as long as a memory, as recordings in the neocortex suggest it decays more slowly (Trepel and Racine, 1998). One suggested mechanism for LTP persistence involves CaMKII and its autophosphorylation (Lisman and Goldring, 1988). New subunits can assemble with the activated holoenzyme and undergo inter-subunit autophosphorylation, suggesting that the CaMKII-mediated synaptic potentiation (ie. “the memory”) can be maintained without further activation of the enzyme (Lisman and Raghavachari, 2015; Stratton et al., 2014). Other proposed mechanisms for LTP

maintenance involve PKMζ, which is the independent catalytic domain of PKCζ (Protein kinase C, zeta), lacking an autoinhibitory regulatory domain of the full-length PKCζ, which makes it constitutively and persistently active, without the need of a second messenger (Pastalkova et al., 2006) . However, studies with PKMζ knockout mice show that LTP and memory are normal (Lee et al., 2013; Volk et al., 2013), reducing the enthusiasm for this hypothesis, despite a recent potential explanation for the contradictory data (Tsokas et al., 2016). Lastly, Kausik Si and Eric Kandel have proposed that prion-like proteins, in particular cytoplasmic polyadenylation element-binding protein (CPEB) are involved in memory maintenance (Si and Kandel, 2016), with evidence suggesting CPEB can acquire a prion-like state and control protein synthesis at the synapse, thereby stabilizing long-lasting changes (Fioriti et al., 2015; Li et al., 2016; Si et al., 2003).

However, due to the high volatility of synaptic connections (Mongillo et al., 2017; Xu et al., 2009), it is possible that other processes besides synaptic potentiation take over to maintain weeks-old memories, after LTP has decayed (Abraham, 2003; Abraham et al., 1995). Recent work has demonstrated that epigenetic modifications are triggered by synaptic plasticity, and that perturbation of epigenetic mechanisms cause impairments in memory storage (Korzus et al., 2004; Mews et al., 2017). Moreover, treatment with histone deacetylase inhibitors, which increase levels of histone acetylation, enhances the formation of long-term memories (Levenson and Sweatt, 2005). Given this evidence and their high stability capacity, epigenetic processes have become a strong candidate mechanism behind long-term memory storage (Kyrke-Smith and Williams, 2018; Landry et al., 2013; Levenson and Sweatt, 2005). Another hypothesis states that long-term memories are stored in the extracellular matrix surrounding neurons, the perineuronal net (Tsien, 2013). Its disruption affects synaptic

plasticity, and memory recall (Wang and Fawcett, 2012), and evidence suggest that its molecular components are long lived (Tsien, 2013). Although these hypotheses propose mechanisms that go beyond synaptic modifications, all of them recognize the central role of synaptic plasticity in memory storage.

1.4. Probing engrams by manipulation of synaptic plasticity

As proposed in this thesis, the best method for engram identification involves manipulation of learning-induces changes, as this is the core of memory storage. As discussed above, synaptic plasticity, including LTP, is the most likely mechanism involved in memory storage, and likely supported by additional mechanisms for long-term memory maintenance. Besides the correlational evidence (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Tsvetkov et al., 2002; Whitlock et al., 2006), there only are a few reports that investigate a causal role of synaptic plasticity in memory storage, which I will review below.

In mammals, most of these studies focused in tone fear conditioning in rodents (Pape and Pare, 2010). In this simple paradigm, an emotionally neutral auditory tone (CS) is paired with foot shocks (US) so that the animal learns to be afraid of the tone. Studies have shown that auditory fear conditioning induces long-lasting potentiation of auditory inputs from the Medial Geniculate Nucleus (MGN) and from the Auditory Cortex (AuC) in the Lateral Amygdala (LA) (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). Furthermore, fear conditioning occludes LTP-induced enhancement of synaptic transmission in the cortical pathway to the LA (Tsvetkov et al., 2002), and promotes AMPAR insertion into the postsynaptic neurons in LA

(Rumpel et al., 2005), which suggests that LTP is the key mechanism behind CS-US association by fulfilling the first criteria to identify an engram (detectability, or correlation).

Recent studies have tried to directly manipulate LTP to address its causal role in tone fear conditioning. First, Nabavi and colleagues (2014) injected adeno-associated virus (AAV) expressing a variant of the light-activated channel ChR2, oChIEF, that can respond faithfully to 50–100 Hz stimuli (Lin et al., 2009), into the MGN nucleus and AuC. Light stimulation at 1 Hz in LA, which they demonstrate to induce LTD, impairs the conditioned response, measured in a lever press paradigm combined with tone fear conditioning (Nabavi et al., 2014). Moreover, light stimulation at 100 Hz in LA, which they demonstrate to induce LTP, rescues the conditioned response. However, the authors were not able to generate a *de novo* fear association by simply potentiating the auditory inputs, which could imply the requirement of other forms of plasticity besides LTP (Johansen et al., 2014) such as US-specific plasticity in downstream circuits (Herry and Johansen, 2014; Tovote et al., 2015). In order to achieve ensemble specificity, Kim and Cho (2017) used a c-fos-CreER^{T2} mouse line in combination with a similar strategy for labeling auditory inputs from MGN and AuC with channelrhodopsin. They used a tone discrimination paradigm to test whether learning-induced potentiation in LA is specific to CS+ (tone paired with shock) inputs, in comparison to CS- (different tone not paired with shock). They found that potentiation is specific to CS+ inputs and that LTD-inducing 1 Hz optogenetic stimulation of CS+ inputs impairs the memory. This result fulfills criterion #2 for identification of an engram, showing a causal role of potentiation of CS+ inputs in tone fear conditioning (Kim and Cho, 2017). More recently, another study had similar findings, providing additional evidence for this criterion (Abdou et al., 2018). This study also found that 100 Hz optogenetic potentiation of auditory inputs to LA labeled during unpaired tone conditioning

was sufficient to produce a *de novo* memory specific to the CS+ (Abdou et al., 2018). Interestingly, they did not get the same result if only the CS+ was labeled (with no shock) suggesting three possibilities: (1) the US exposure generates specific plasticity that is required for conditioning, as suggested above; or (2) their effect is actually due to a hybrid memory association (Garner et al., 2012) formed between the context memory, which was naturally formed as a consequence of the unpaired tone conditioning protocol, and the artificial potentiation of CS+ inputs ; or (3) there are US-specific neurons in the MGN and AuC, and their inputs need to be potentiated for memory formation, which is unlikely. This study provided the best evidence so far for the sufficiency of LTP onto auditory inputs in LA on tone fear conditioning, partially fulfilling criterion #3.

In addition to the studies manipulating plasticity in tone fear conditioning, one study probed the causal role of synaptic plasticity in the motor cortex during motor learning. Hayashi-Takagi and others developed a tool to label potentiated spines and induce their selective shrinkage with light. Using this tool, they show that the potentiated spines in superficial layers of the motor cortex are required for motor performance after training on the rotarod or a balance beam (Hayashi-Takagi et al., 2015). This study provided evidence that synaptic plasticity is also required for storage of a different kind of memory (criterion #2).

All the studies mentioned above were published after the work for this thesis had started. Our work expands on these previous studies, focusing on providing more robust evidence towards criterion #3 - mimicking plasticity to test sufficiency -, which has not been properly dissected by any known study so far.

1.5. A context fear conditioning engram

This thesis focuses on contextual fear conditioning, in which a box with distinct spatial, visual and odor cues is paired with a foot shock, generating a memory between a complex CS, which is thought to involve processing in many cortical areas and the hippocampus, and the US. Indeed, at least two types of memories are necessary for context conditioning: context encoding, i.e. learning about the context itself, and conditioning, i.e. CS- -US association (Maren et al., 2013). The use of appropriate controls, such as exposure to context only, and also overnight habituation before shock exposure, which prevents context conditioning (Impey et al., 1998), are crucial to discriminate between context encoding and conditioning, as well as US exposure-mediated plasticity.

In contextual fear conditioning, lesion and pharmacological studies support a role for many brain areas, including the hippocampus, retrosplenial cortex, prefrontal cortex, and basolateral amygdala (BLA) (Corcoran et al., 2011; Gale et al., 2004; Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Quinn et al., 2005), and studies using optogenetic activation of c-fos labeled neuronal ensembles demonstrate that artificial reactivation of learning specific ensembles in each of these areas can induce an apparent recall event (Cowansage et al., 2014; Gore et al., 2015; Kitamura et al., 2017; Liu et al., 2012). While these studies define the circuitry controlling the behavior and suggest a widely distributed network, they do not identify the sites of plasticity that instantiate the memory**.**

The observation of plasticity in learning-activated (c-fos+) neurons after contextual fear conditioning has been recently described in the hippocampus (Choi et al., 2018; Ryan et al., 2015). Choi and colleagues developed a tool to visualize synapses between learning-activated

ensembles in different regions and showed that the number of CA3-CA1 synapses was higher in animals that underwent contextual fear conditioning compared to a box exposure control (Choi et al., 2018). Although these studies provide evidence correlating synaptic plasticity to context conditioning (criterion #1), no study so far has directly tested the role of synaptic plasticity in contextual fear conditioning (criterion #2). Likewise, no study so far has provided robust evidence that some form of learning-induced change is sufficient for context conditioning (criterion #3). Ohkawa and colleagues (2015) came close, demonstrating that, artificial coincidental optogenetic activation of CS and US neurons in the dorsal hippocampus and BLA is sufficient to generate an aversive memory to the tagged box, which otherwise would not be formed (Ohkawa et al., 2015). However, the lack of known direct connections between these regions preclude the investigation of the mechanism behind this artificial stimulation and put into question if it parallels a natural learning association.

A recent study looked at context reward conditioning and found that LTP-inducing 100 Hz optogenetic stimulation of ventral hippocampal inputs to the nucleus accumbens shell is sufficient to drive conditioned-place preference (LeGates et al., 2018), providing some evidence regarding criterion #3 (Mimicking plasticity to test sufficiency). However, this study did not show if these inputs were potentiated by natural cues, or if impairment of this plasticity was necessary for reward behavior (criteria #1 and #2).

The role of amygdala plasticity in contextual fear learning is even less characterized, despite being an important part of the fear center (Tovote et al., 2015). A few studies have shown that the infusion of NMDAR blockers in BLA during conditioning impairs memory recall (Matus-Amat et al., 2007; Rodrigues et al., 2001). However, these experiments lack cellular specificity and the drugs used might have off-target effects. Evidence of learning-

induces changes in BLA after contextual conditioning is scarce. One recent study reported an increase in synaptic strength in LA after contextual conditioning (Butler et al., 2018), although the authors do not properly control for the possibility of CS or US-only mediated plasticity. Interestingly, in many of the studies examining learning-induced plasticity in the LA/BLA after tone fear conditioning, the behavioral training would be expected to produce a contextual fear memory in addition to tone conditioning, yet the synaptic changes observed were only dependent on tone-shock pairing contingencies (McKernan and Shinnick-Gallagher, 1997; Namburi et al., 2015; Tsvetkov et al., 2002). This could reflect either a lack of amygdala-based plasticity underlying context-fear associations or a sparse context representation that was not captured by recording from randomly targeted neurons.

1.6. Hypothesis and main goals

As discussed above, despite substantial progress over the last few years, there are only a few reports that directly tested a causal role of synaptic plasticity in memory (Abdou et al., 2018; Hayashi-Takagi et al., 2015; Kim and Cho, 2017), especially for tone fear conditioning. These studies found that plasticity in auditory inputs to the lateral amygdala is required for tone conditioning (Abdou et al., 2018; Kim and Cho, 2017), but not necessarily sufficient (Nabavi et al., 2014). Given that synaptic potentiation in amygdala is required for tone conditioning, and that the role of amygdala plasticity in contextual fear conditioning is less characterized, we chose to systematically investigate it. Several reasons indicate that the BLA is a great candidate region to store the fear conditioning engram: (a) BLA is an emotional center, especially for the processing of aversive stimuli (Bechara et al., 1995; Feinstein et al., 2011); (b) it receives inputs

representing both the context (CS) (Redondo et al., 2014; Xu et al., 2016) and the shock (US) (Grewe et al., 2017; Romanski et al., 1993), suggesting that the CS-US association might take place in the BLA; (c) lesions to the BLA impair contextual fear conditioning and recall (Gale et al., 2004; Phillips and LeDoux, 1992), as well as injection of drugs that affect synaptic plasticity (Matus-Amat et al., 2007); (d) optogenetic reactivation of US-tagged neurons in the BLA induces apparent memory recall (Gore et al., 2015). Therefore, the main hypothesis of this thesis is that the engram for contextual fear conditioning relies on synaptic potentiation of learning-activated neurons in the BLA. In chapter 2, I describe how I tested all three criteria for identifying an engram, the main goals of this thesis:

1. Is synaptic potentiation detected specifically in learning-activated neurons of the BLA following context conditioning?
2. If so, is this plasticity required for memory recall? Does the erasure of learning-induced synaptic potentiation impair memory recall?
3. Is this plasticity sufficient to generate a CS-US association? Does the artificial induction of synaptic potentiation in BLA create a *de novo* aversive memory?

In chapter 3, I discuss the main findings and their implications on our understanding of learning and memory. Finally, chapter 4 describes the methods used to conduct the work described on this thesis.

Chapter 1, in part, has been submitted for publication in Science, 2019. Cardozo, L.M.; Dillingham, B.C.; de Sousa, A.F.; Dang, W.; Job, N.; Yoo, E.J.; Ranamukhaarachchi, S.K.; Yuan, Q.; Mayford, M. Generation of Context Fear Memory through Localized Synaptic Potentiation. The dissertation author was the primary investigator and author of this material.

Chapter 2: Results

All approaches at a higher level are suspect
until confirmed at the molecular level.

Francis Crick
What Mad Pursuit, 1988

In this chapter, I will describe how we tested the role of synaptic potentiation in the basolateral amygdala (BLA) on context fear conditioning. We developed an approach to systematically test the criteria for engram identification, as outlined in Chapter 1.

2.1. Context fear conditioning induces synaptic potentiation specifically in learning-activated neurons of the BLA

To test whether context conditioning induces circuit-specific synaptic changes in the BLA, we used the c-fos-shEGFP transgenic mouse line (Figure 1A), which expresses a short-half life EGFP under the control of the c-fos promoter (Reijmers et al., 2007). Mice underwent associative fear conditioning and were either tested for freezing memory 24 hours later or sacrificed after 90 minutes followed by whole-cell recordings in a BLA brain slice preparation (Figure 1, B and C). Synaptic responses of GFP+ (c-fos+) and GFP- neurons were examined with targeted recordings in the BLA while stimulating internal capsule input fibers (Figure 2A). We recorded α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) / N-methyl-D-aspartate receptor (NMDAR) ratios as a measure of excitatory synaptic strength (Namburi et al., 2015; Rumpel et al., 2005), and found that fear conditioning produced an increase in AMPAR/NMDAR ratio that was limited to the GFP+ neurons (Figure 2B),

consistent with activity dependent-potentiation and as seen previously in the LA with tone conditioning (Abdou et al., 2018; Kim and Cho, 2017).

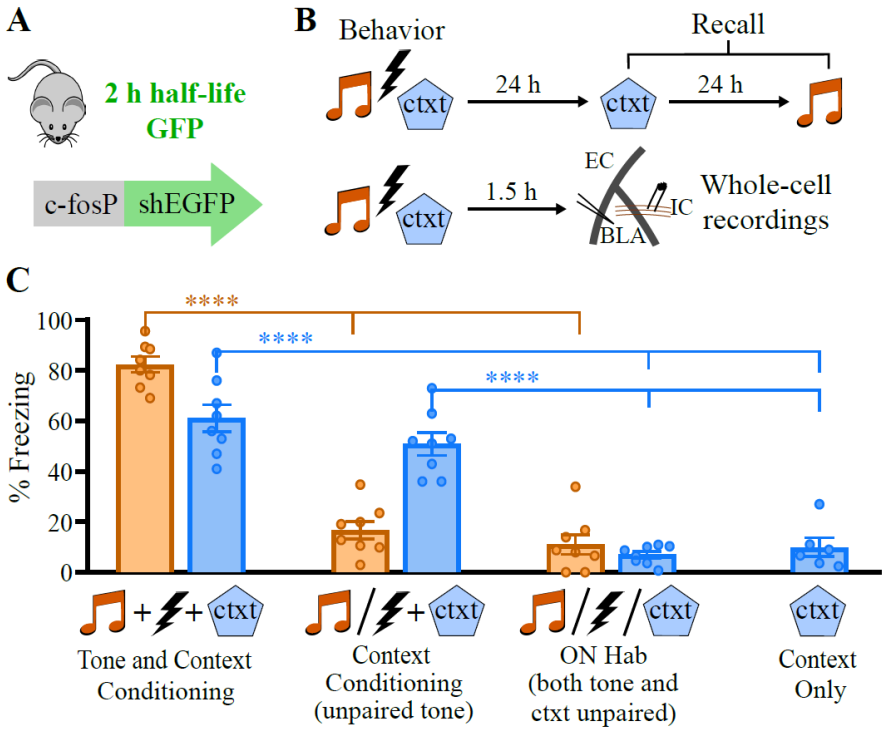


Figure 1. Experimental setup to test whether synaptic potentiation is observed in BLA following contextual fear conditioning.
(**A**) The c-fos-shEGFP transgenic mouse, expressing a short half-life (sh)EGFP under the control of the c-fos promoter. (**B**) Experimental design in which the c-fos-shEGFP mouse was either used for whole-cell recordings or memory recall tests after different behavior treatments. Ctxt: context; EC: external capsule; IC: internal capsule; BLA: basolateral amygdala. (**C**) Freezing response to the context and/or tone measured 1 day after correspondent behavior treatment. ON Hab: Overnight Habituation group. N = 8 per group, except in "Context only" group, in which n = 6. ****P < 0.0001, one-way ANOVA with Tukey test. Graph bars show mean +/- SEM.

However, unlike previous studies (McKernan and Shinnick-Gallagher, 1997; Namburi et al., 2015; Tsvetkov et al., 2002), we also observed an increase in AMPAR/NMDAR ratio during unpaired tone conditioning (i.e. conditioning to context only), suggesting synaptic potentiation occurs in BLA after context conditioning (Figure 2B). The observed increase in AMPAR/NMDAR ratio is likely due to associative learning as a protocol that does not induce

context conditioning, with overnight habituation to the context (Impey et al., 1998) (ON hab; Figure 1C), fails to induce an AMPAR/NMDAR ratio increase (Figure 2B). Despite the increase in AMPAR/NMDAR ratio after context conditioning, GFP+ neurons do not differ in spike number, latency to spike, resting-membrane potential or paired pulse ratio (Figure 3).

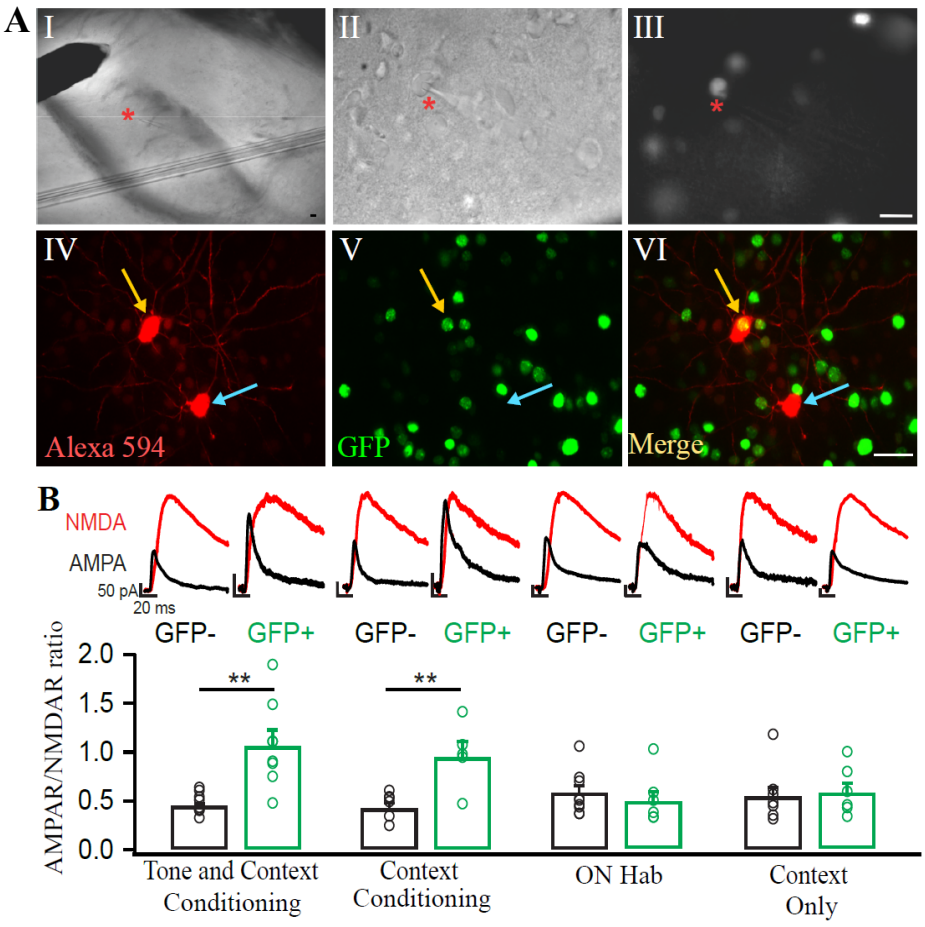


Figure 2. Context learning induces synaptic potentiation specifically in learning-activated neurons.

(**A**) Whole-cell recording design. Panel I shows stimulating electrode over Internal Capsule fiber bundle. II and III illustrate ongoing whole-cell recording (red asterisk) of a GFP+ neuron. IV-VI are confocal images of neighboring neurons filled with Alexa 594 (red) during recordings: the GFP+ neuron is highlighted by a yellow arrow, while the GFP- is highlighted by a cyan arrow. Scale bars: 50µm. These images were taken from a slice of an Arc-tTA x TetO-H2B-GFP mouse (F). (**B**) AMPAR/NMDAR ratio of GFP+ versus GFP- BLA neurons from acute brain slices of Fos-shGFP mice sac'ed 90 min after behavior treatment. An increase in AMPAR/NMDAR ratio was only observed after successful conditioning and was restricted to GFP+ neurons. ON Hab: Overnight Habituation group. N = 6-9 per group. **P < 0.01, unpaired t test. Graph bars show mean +/- SEM.

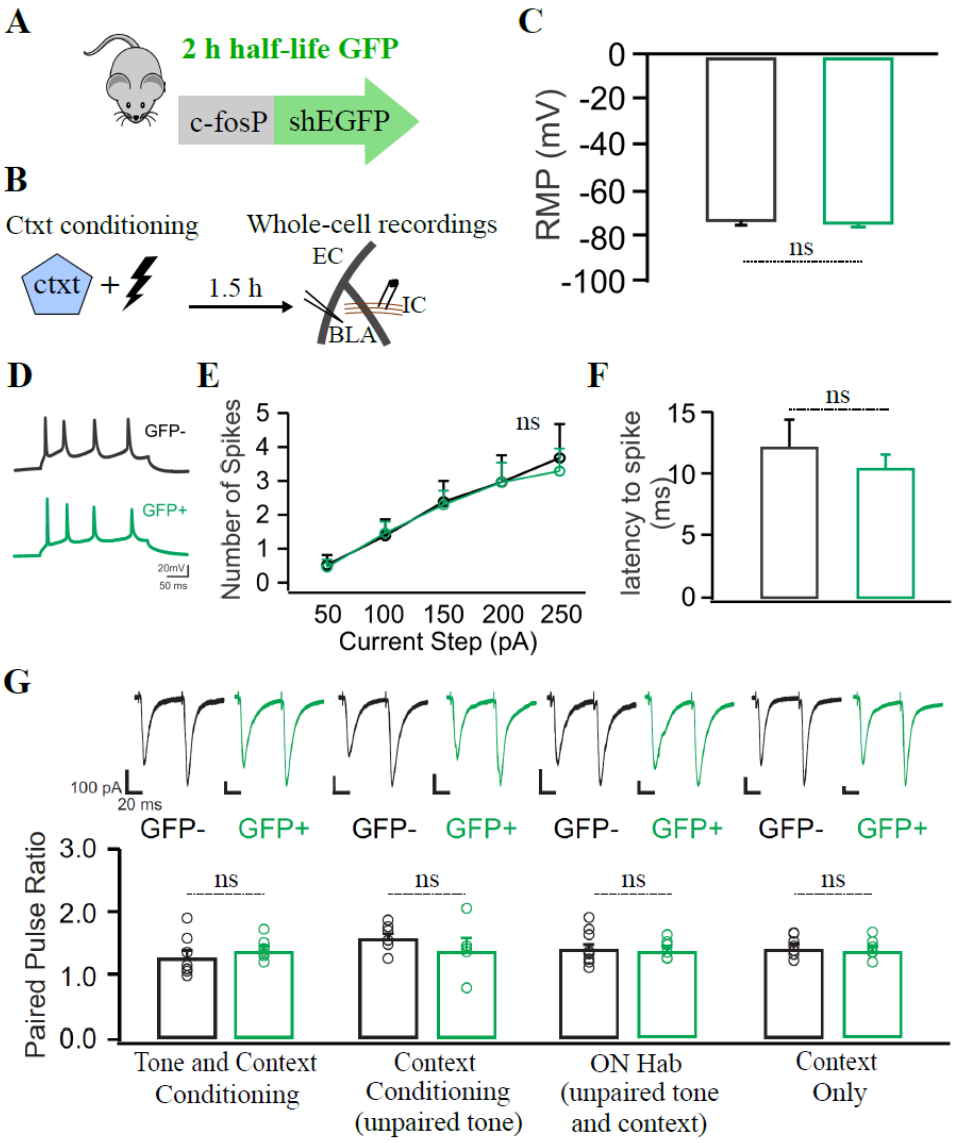


Figure 3. c-fos+ neurons tagged during training do not differ in intrinsic excitability, resting membrane potential or paired pulse ratio.

(**A**) c-fos-shEGFP mouse line, which expresses a short half-life (sh)EGFP under the control of the c-fos promoter. (**B**) Experimental design, in which the c-fos-shEGFP mouse was used for whole-cell recordings after context conditioning. (**C**) The resting membrane potential (RMP) of GFP+ (green) and GFP- (black) neurons are similar. (**D**) Example of evoked train of action potentials with 250pA current injection. (**E**) Both GFP+ and GFP- neurons fire similar number of action potentials in response to given current injections at the internal capsule. (**F**) The latency to spike between GFP+ and GFP- neurons is also similar. N = 6-7 neurons per group in (C) to (F). (**G**) The paired-pulse ratio of GFP+ and GFP- neurons show no evidence for significant pre-synaptic plasticity across the behavior groups tested in Figure 1C. N =6-9 neurons per group. Unpaired t tests [(C), (E) to (G)], ns = not significant. Graph bars show mean +/- SEM.

To examine the persistence of plasticity in BLA neurons after context conditioning, we employed the double transgenic Arc-tTA/TetO-H2BGFP mouse line (Figure 4A), in which a long-lasting histone-bound GFP is expressed in Arc+ cells (Tayler et al., 2013). Active neurons were tagged with GFP at the time of learning and acute slices were prepared for whole-cell recordings 7 days later (Figure 4B). We found that the AMPA/NMDAR ratio increase in learning-activated neurons persists for at least 7 days (Figure 4C), however potentiation was reversed in a group that received extinction training (Figure 5). Re-conditioning animals that had undergone extinction reinstates the AMPAR/NMDAR ratio increase in the original GFP+ neuronal population (Figure 5). Together, these results suggest that, following contextual fear conditioning, learning-induced synaptic potentiation in BLA neurons is circuit-specific, long-lasting, and is modulated by post-learning experience.

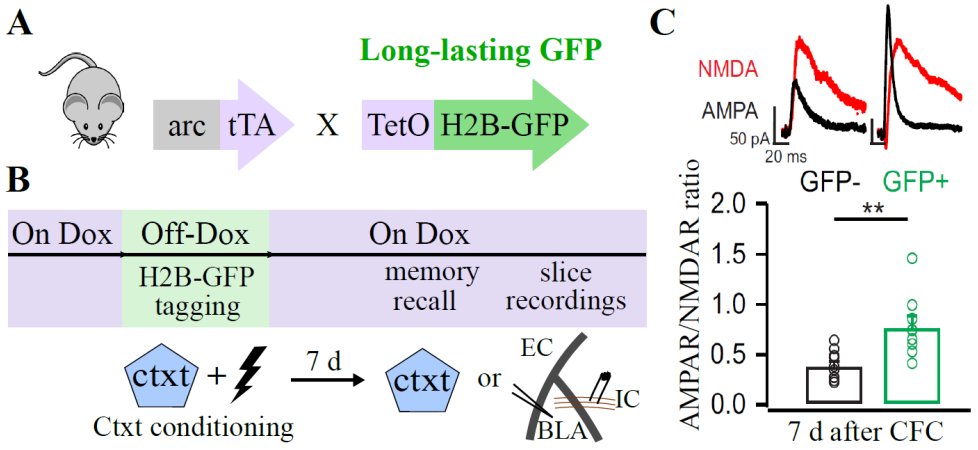


Figure 4. Synaptic potentiation following context conditioning lasts for at least 7 days.
(**A**) Arc-tTA x TetO-H2BGFP double transgenic mouse, in which the tetracycline transactivator (tTA) was knocked in the Arc gene and controls the expression of the long-lasting histone-bound GFP. (**B**) Experimental design illustrating the tagging window of the Arc-tTA/TetO-H2B-GFP mouse line, controlled by the presence of Doxycycline (Dox) in the food. Tagged neurons were recorded 7 days after. Correspondent behavior result can be found on Figure 5. (**C**) Specific AMPAR/NMDAR ratio increase on GFP+ neurons following conditioning is maintained for at least 7 days. N = 8-9 per group. **P < 0.01, unpaired t test. Graph bars show mean +/- SEM.

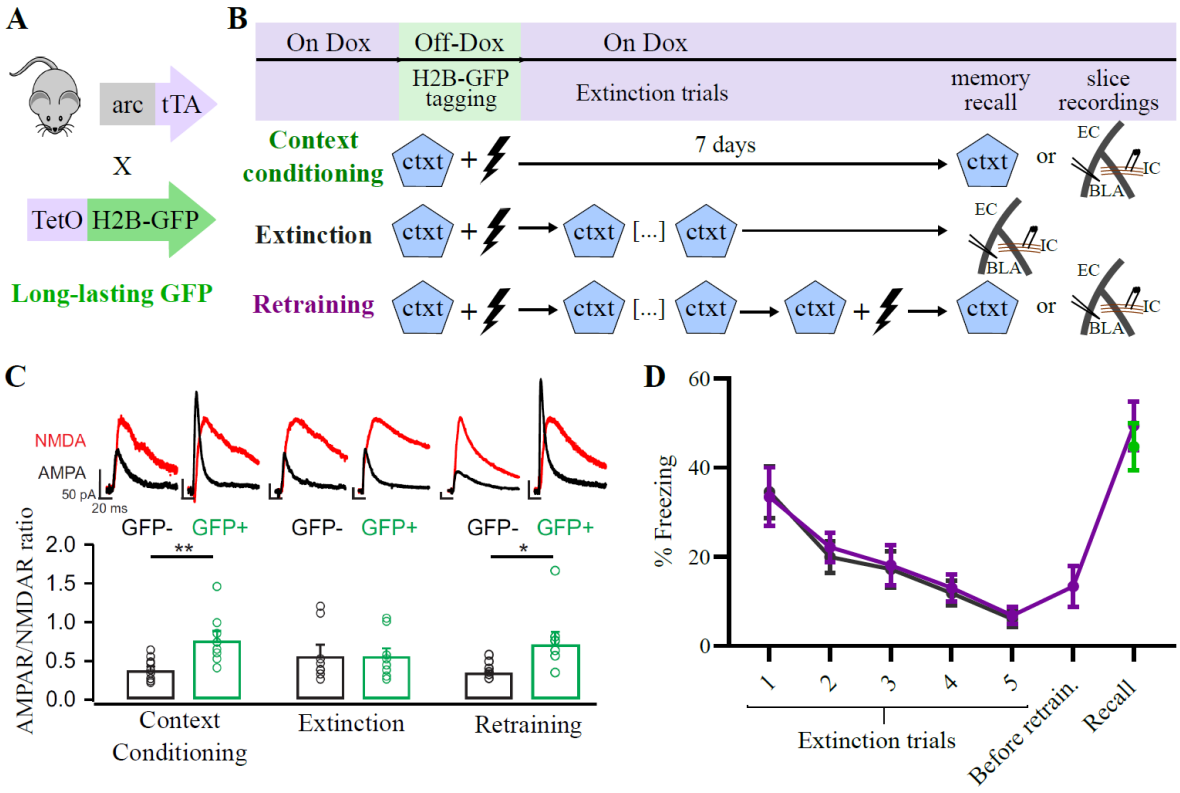


Figure 5. Long-lasting increase in AMPAR/NMDAR ratio in Arc+ neurons tagged during training is reversed by extinction and reinstated by retraining.

(**A**) Arc-tTA x TetO-H2BGFP double transgenic mouse, in which the tetracycline transactivator (tTA) was knocked in the Arc gene and controls the expression of the long-lasting histone-bound GFP. (**B**) Experimental design, in which the Arc-tTA x TetO-H2BGFP mouse was used to tag Arc+ cells during training with a long-lasting histone-bound GFP for subsequent behavior or recordings 7 days later. Animals submitted to context extinction were exposed daily for 5 days to conditioned context for 30 min/day. (**C**) Freezing levels across the extinction trials and after retraining, which restores freezing to levels compared to animals that did not undergo extinction. Green: context conditioning group; black: extinction group; purple: Retraining group. N = 8 mice per group. (**D**) Extinction reverses long lasting increase in AMPAR/NMDAR ratio of learning-activated neurons (GFP+), while re-training reinstates the increase in AMPAR/NMDAR ratio in the same population of neurons activated during the original conditioning. N = 6-9 neurons per group. *P < 0.05, **P < 0.01, unpaired t test (C). Graph bars show mean +/- SEM.

2.2. Disruption of learning-induced synaptic potentiation in BLA impairs contextual fear memory

To test whether the synaptic potentiation in learning-induced BLA neurons represents a necessary component of the context fear memory trace, we used a biochemical approach to reverse the plasticity. CaMKIIα is a critical component in the induction of synaptic plasticity, with mutant forms of the kinase known to either induce long term-potentiation (LTP) or long-term depression (LTD) (Pi et al., 2010). We used a c-fos driven-inducible Cre recombinase mouse line (FDC mouse) (Dillingham et al., 2019) combined with AAV-based gene delivery to introduce the LTD inducing CaMKIIα-T286D (CK2-D) mutant (Pi et al., 2010) into BLA neurons activated with learning (Figure 6A). In the FDC mouse, Cre-recombinase is regulated by trimethoprim-induced protein stabilization, providing a ligand-gated time window for Cre activity of approximately 4 hours (Dillingham et al., 2019; Sando III et al., 2013). Using this system, we induced expression of CK2-D and a GFP marker in BLA neurons activated during fear conditioning (Figure 6, B and C) and tested the memory on the same day, before CK2-D expression, and 7 days after CK2-D induction (Figure 6D), when it has reached peak expression (Figure 7).

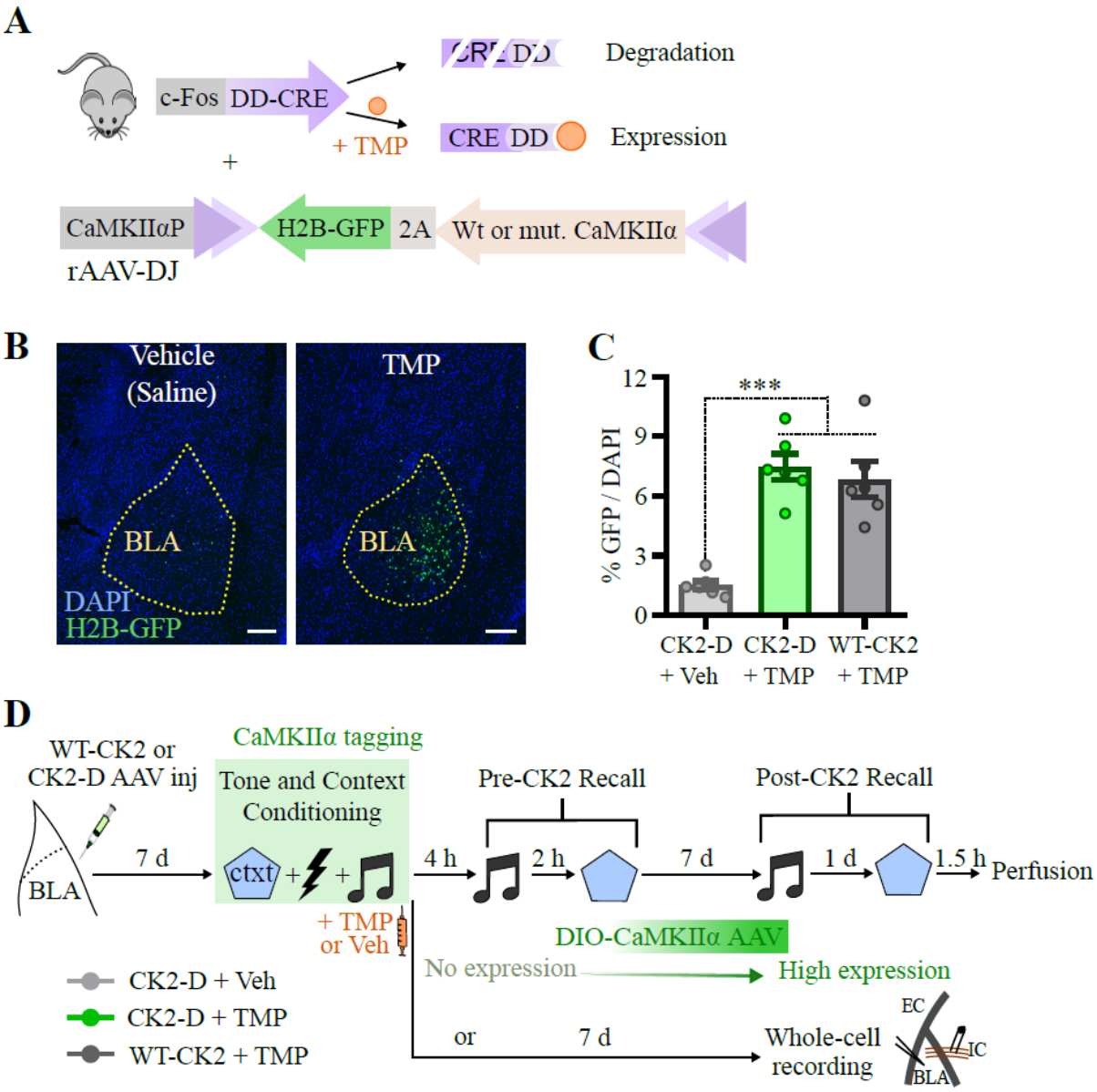


Figure 6. Experimental strategy to test whether reversal of learning-induced plasticity impairs memory.

(**A**) rAAV-DJ carrying a DIO-(wild-type or mutant) CaMKIIα-2A-H2BGFP construct was injected bilaterally in the BLA of c-fos-DD-Cre (FDC) mice**,** in which Cre-recombinase is fused to a destabilizing domain (DD), which leads to protein degradation. The drug trimethoprim lactate (TMP) stabilizes the complex, allowing Cre to activate the CaMKIIα construct along with the H2B-GFP marker in c-fos+ BLA neurons. (**B**) and (**C**) Representative coronal BLA sections showing H2B-GFP induction 14d after TMP versus vehicle (saline) IP injection, quantified in (C). N = 6 mice per group. Scale bar: 200µm. (**D**) Experimental design to test whether CK2-D impairs memory recall. ***P < 0.001, one-way ANOVA with Tukey test. Graphs show mean +/- SEM.

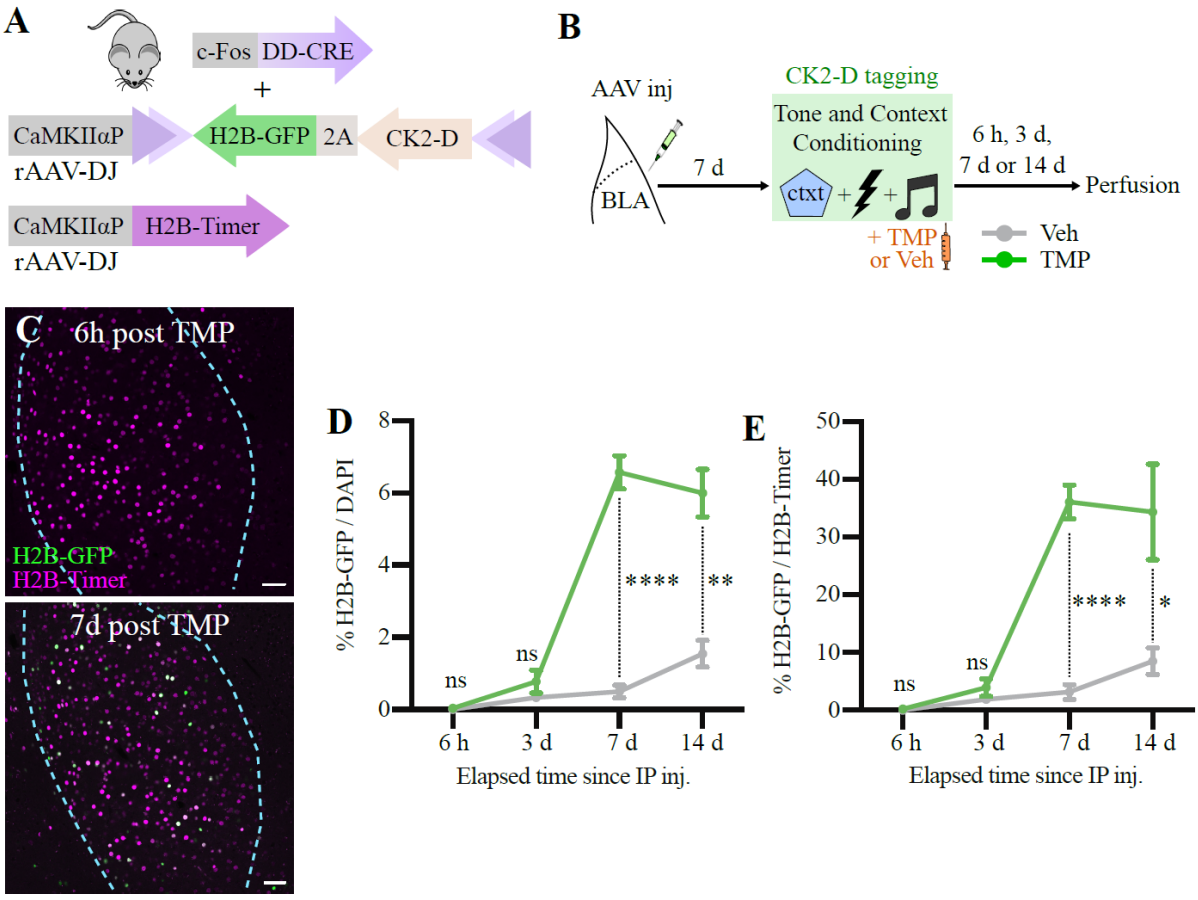


Figure 7. CK2-D expression is minimal at 6 hours post TMP injection, peaking 7 days after.
(**A**) rAAV-DJs carrying a DIO-CK2 mut. construct and a red marker (H2B-Timer) construct were co-injected bilaterally in the BLA of FDC mice. (**B**) Experimental design to determine the time course of CamKIIα-T286D (CK2-D) expression after tagging during fear conditioning. (**C**) Representative images of coronal BLA sections 6 hours and 7 days after fear conditioning followed by IP injection of TMP. BLA is highlighted within dashed cyan lines. Scale bar: 50 µm. (**D**) The percentage of total BLA cells (DAPI) that express CK2-D (H2B-GFP) after TMP or Veh injections reaches its peak 7 days after TMP injection. (**E**) A similar result is found normalizing the number of CK2-D cells (H2B-GFP) by the number of infected cells (H2B-Timer), which takes into account injection coverage variation. N = 4 mice per group per time point, except for 6 hours, where N = 3 (D) and (E). ****P < 0.0001,**P < 0.01, *P < 0.05, ns, not significant, unpaired t test. Graph bars show mean +/- SEM.

CK2-D expression in c-fos+ BLA neurons tagged during training reverses the long-lasting increase in AMPAR/NMDAR ratio promoted by learning [(Figure 8A), compare with Figure 4C], suggesting reversal of learning-induced synaptic potentiation. Behaviorally, CK2-D expression in c-fos+ BLA neurons tagged during training impairs both tone and context memories, while the control group, injected with wild-type CaMKIIα (WT-CK2) AAV had intact memories, suggesting the effect is not due to overexpression of CaMKIIα (Figure 8, B and C). Staining for endogenous c-fos during memory retrieval showed that the reactivation of neurons tagged during training (GFP+), which has been shown to correlate with memory retrieval (Reijmers et al., 2007; Tayler et al., 2013), was impaired by CK2-D expression (Figure 9, A and B), suggesting an inability to access these ensembles during recall.

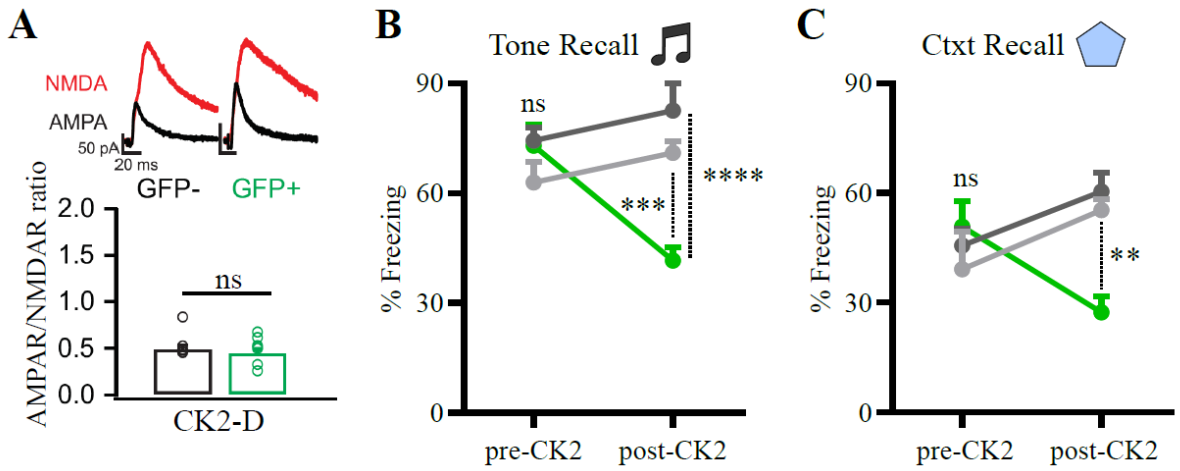


Figure 8. CaMKIIα-T286D (CK2-D) expression in c-fos+ BLA neurons reverses learning-induced plasticity.

(A) CK2-D expression in c-fos+ BLA neurons tagged during context conditioning reverses learning-induced AMPAR/NMDAR ratio increase. No tone conditioning/exposure was performed in order to allow for correct interpretation of recordings. N = 7-8 neurons per group. (**B**) and (**C**) CK2-D expression in c-fos+ BLA neurons tagged during training impairs long-term memory, as opposed to WT-CK2. N= 6 mice per group. **P < 0.01, ***P < 0.001, ****P < 0.0001, ns, not significant; unpaired t-test (A) or two-way RM ANOVA with Tukey test [(C) and (D)]. Graphs show mean +/- SEM.

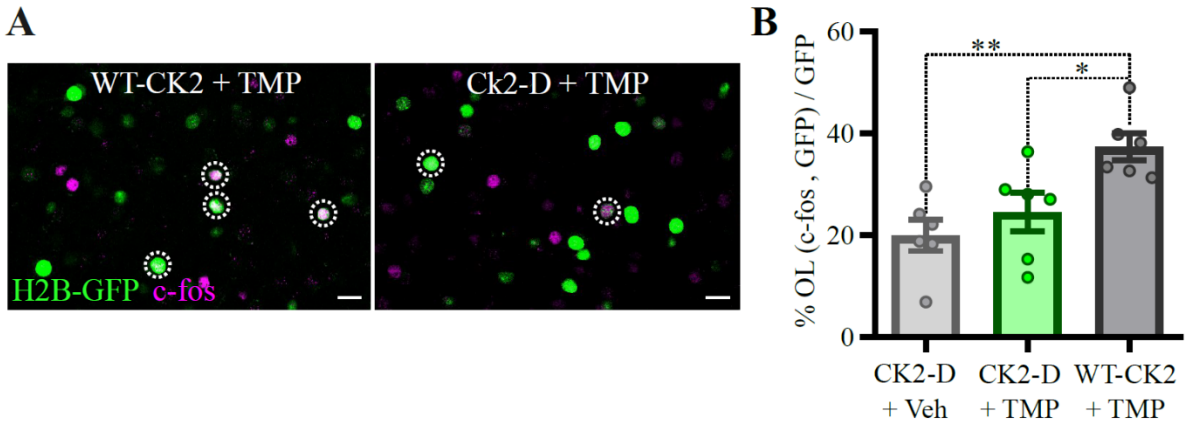


Figure 9. Reactivation of learning ensemble during memory recall is impaired by CK2-D expression.
(**A**) and (**B**) Representative coronal BLA sections showing overlap between activity markers during training (H2B-GFP) and context recall [(c-fos IHC), (H)], quantified in (B). OL: overlap. N = 6 mice per group. Scale bar: 20 μm. P < 0.05, **P < 0.01; one-way ANOVA with Tukey test. Graphs show mean +/- SEM.

Memory recall tests four weeks after training indicates that the CK2-D induced memory impairment is long-lasting (Figure 10). Physiologically, in addition to reversing the learning-induced increase in AMPAR/NMDAR ratio, CK2-D expression also reduces the amplitude of mini-EPSCs without affecting their frequency, paired-pulse ratio or intrinsic excitability (Figure 11 and table 1), which suggests specific reversal of learning-induced synaptic plasticity.

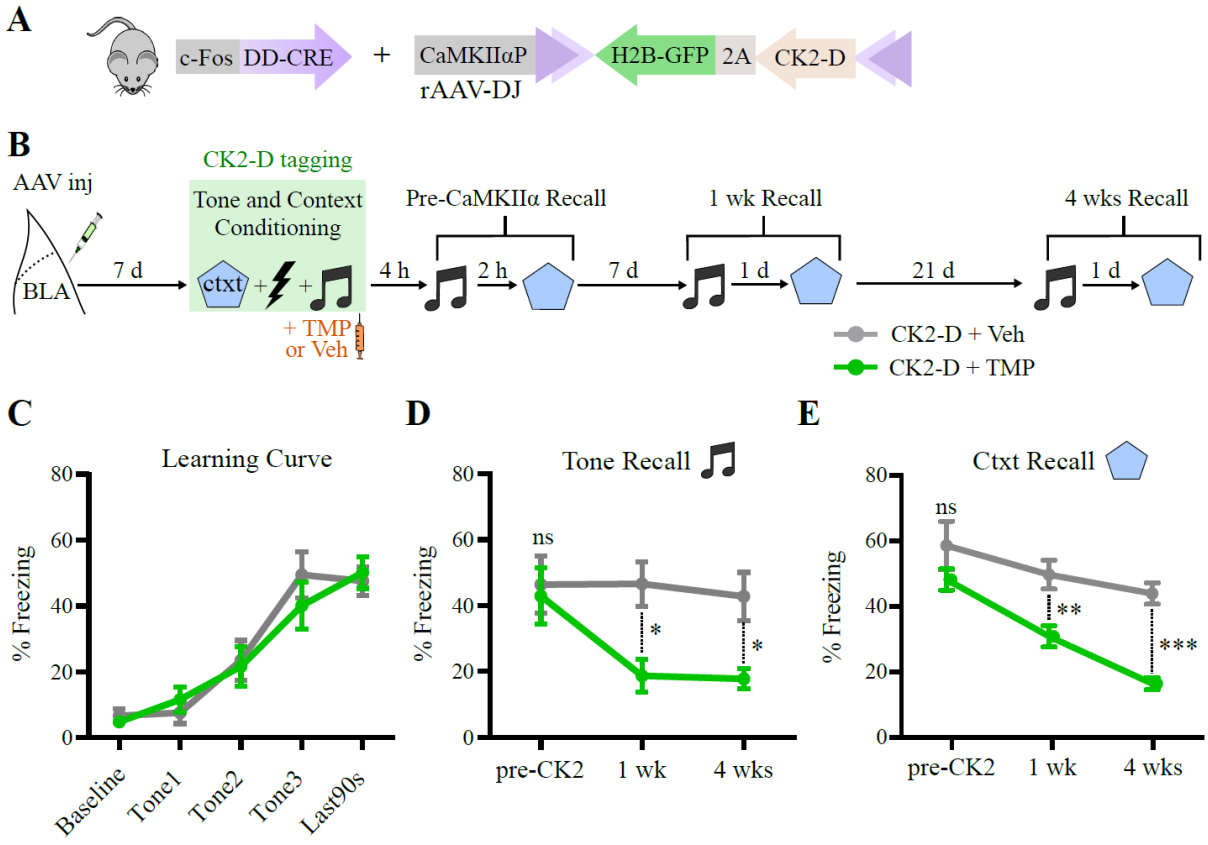


Figure 10. CaMKIIα-T286D (CK2-D) effect in c-fos+ BLA neurons tagged during training is long-lasting.

(**A**) rAAV-DJ carrying a Cre-dependent CK2-D-2A-H2BGFP construct was injected bilaterally in the BLA of FDC mice. (**B**) Experimental design to test the effects of CK2-D expression over time in c-fos+ BLA neurons tagged during fear conditioning. (**C**) Freezing response across training trial, with three tone-shock pairings. As expected, the freezing to the tone increased after pairing with shock. (**D**) and (**E**) CK2-D mediated-memory impairment for tone (D) and context (E) lasts for at least 4 weeks. N = 7 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001, ns, not significant, two-way RM ANOVA with Bonferroni test [(C) to (E)]. Graph bars show mean +/- SEM.

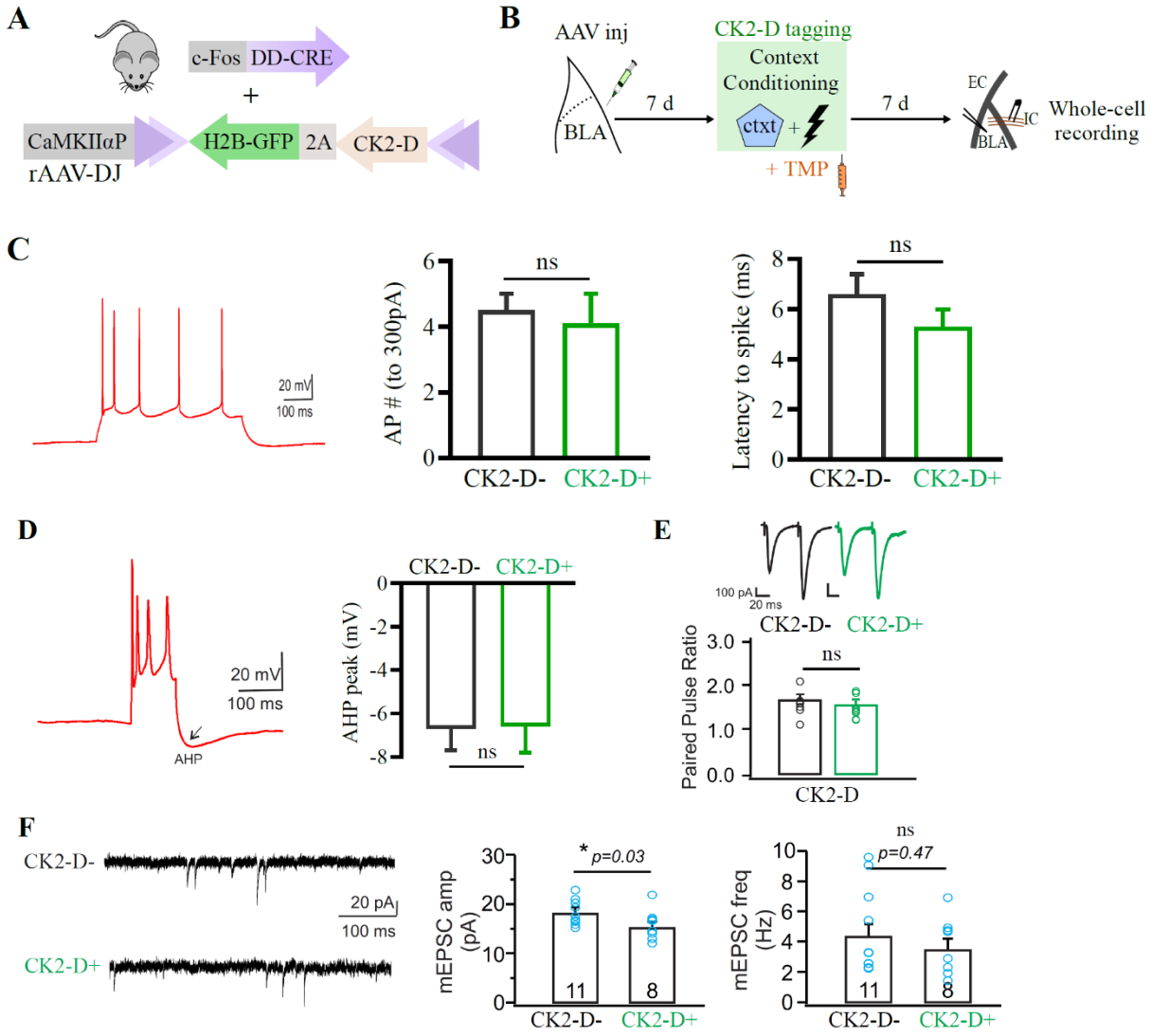


Figure 11. CK2-D expression in c-fos+ BLA neurons tagged during training affects the amplitude of mEPSCs but not the frequency, without altering the intrinsic excitability or paired-pulse ratio. (**A**) rAAV-DJ carrying a Cre-dependent CK2-D-2A-H2BGFP construct was injected bilaterally in the BLA of FDC mice. (**B**) Experimental design to test the effects of CK2-D expression on the intrinsic excitability, paired-pulse ratio and mEPSCs of c-fos+ BLA neurons tagged during context conditioning. (**C**) and (**D**) CK-2D expression does not affect the number of action potentials triggered by 300pA current injection in the internal capsule fibers neither the latency to spike (C), nor the after-hyperpolarization (AHP) peak after a train of 4 or more action potentials (D). N = 16 CK2-D- neurons and 10 Ck2-D+ neurons. Additional measurements regarding these neurons can be found on table S1. (**E**) The paired-pulse ratio of CK2-D+ and Ck2-D- neurons show no evidence for significant pre-synaptic plasticity after training. N = 7-8 per group. (**F**) mEPSCs from CK2-D+ neurons have smaller amplitudes, but no difference in frequency, compared to Ck2-D- neurons, suggesting weakening of synaptic responses. Number of recorded mEPSCs indicated within bars. *P < 0.03, ns, not significant, unpaired t tests. Graph bars show mean +/- SEM.

Table 1. CaMKIIα-T286D (CK2-D) expression in c-fos+ BLA neurons tagged during training does not affect basic electrophysiological properties.
Results of unpaired t tests and the respective P value is included for every corresponding measure. Part of the data described here is plotted on Figure 11. AP: action potential, V resting: resting potential, Rm: Membrane resistance, AHP: afterhyperpolarization.

	CK2-D-	CK2-D+	t	p
	n= 16	*n = 10*		
AP threshold (mV)	-44.3 ± 1.1	-44.4 ± 1.6	0.04	0.97
AP amplitude (mV)	74.7 ± 2.8	72.8 ± 3.7	0.41	0.69
AP half width (ms)	0.96 ± 0.09	0.85 ± 0.08	0.8	0.43
V resting (mV)	-74.1 ± 1.9	-74.3 ± 2.8	0.07	0.94
Rm (Mohm)	222.2 ± 28.9	210.4 ± 32.7	0.26	0.79
AP numbers at 300pA	4.5 ± 0.5	4.1 ± 0.9	0.43	0.67
Latency to spike (ms)	6.6 ± 0.8	5.3 ± 0.7	1.12	0.27
AHP (mV)	6.7 ± 1.0	6.6 ± 1.2	0.09	0.93

To test whether CK2-D-mediated memory impairment was context-specific, we genetically tagged c-fos neuronal ensembles activated in either the conditioning box (box A) or a novel box (box B) one day after training (Figure 12, A and B). Recall in box A 7 days later showed that the memory was impaired only when CK2-D was expressed in box A ensembles (Figure 12, C and D), despite a similar proportion of labeled neurons in both groups (Figure 12E). In summary, these results suggest that synaptic potentiation in learning-activated BLA neurons is a necessary component of the contextual fear memory trace, and that this plasticity is context specific.

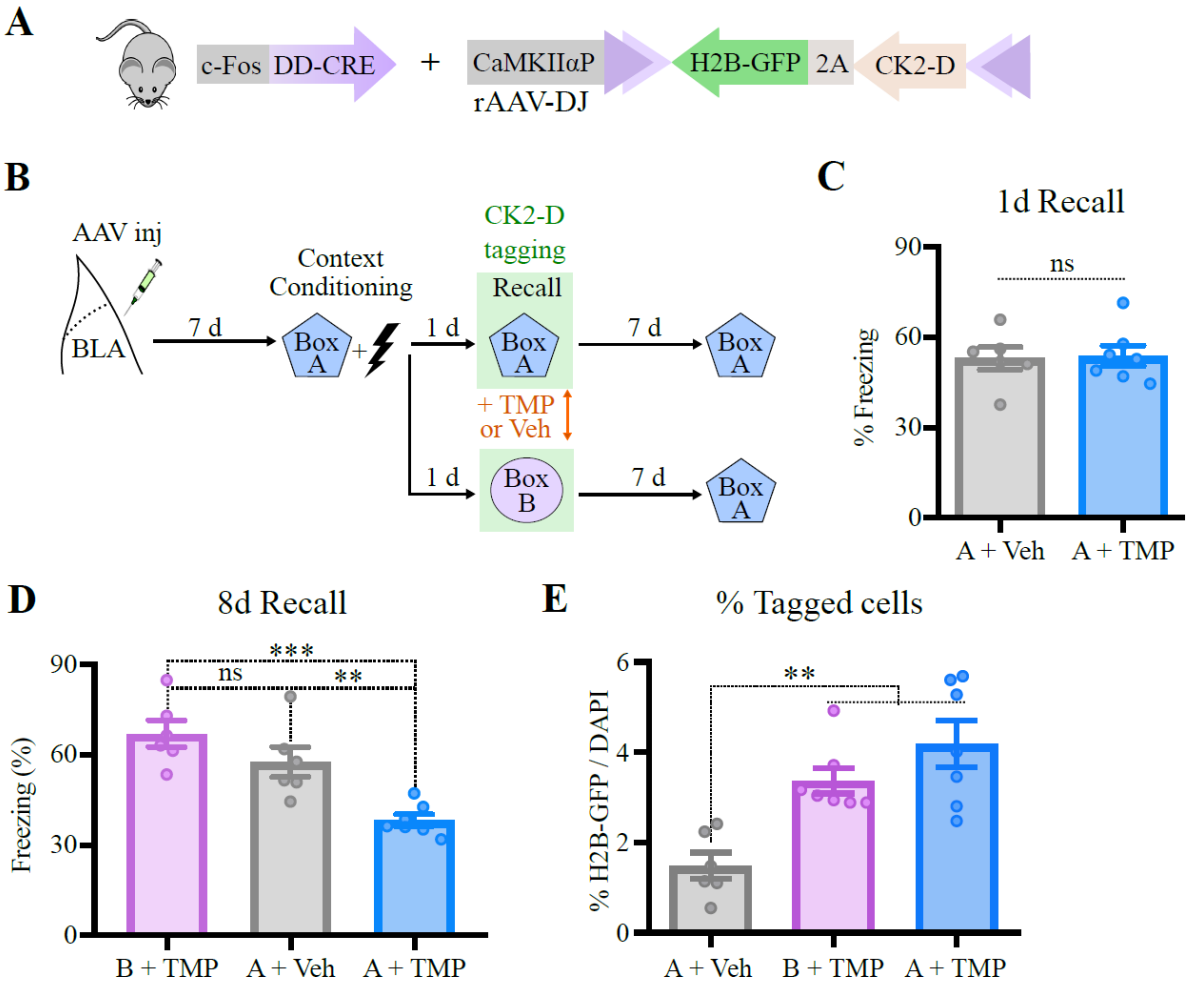


Figure 12. CK2-D mediated-memory impairment in BLA is context specific.
(**A**) rAAV-DJ carrying a Cre-dependent CK2-D-2A-H2BGFP construct was injected bilaterally in the BLA of FDC mice. (**B**) Experimental design to test specificity of effect to the tagged context. N =6-7 per group. (**C**) 1-day recall in box A before CK2-D induction shows that memory was similar between groups. (**D**) CK2-D-mediated memory impairment is specific to tagged context. (**E**) Percent of tagged cells (H2B-GFP+) was similar between group tagged during recall to conditioning box (box A) and exposure to novel box (box B). N = 6 mice per group. **P < 0.01, ***P < 0.001, ns, not significant; unpaired t-test (C) or one-way ANOVA with Tukey test [(D) and (E)]. Graphs show mean +/- SEM.

2.3. Artificial induction of plasticity in CS inputs to US-responsive BLA neurons is sufficient to generate a *de novo* contextual fear memory.

Next, we sought to test whether synaptic potentiation alone in learning-activated BLA neurons is sufficient to produce a context fear association. We attempted to produce a *de novo* associative fear memory by inducing plasticity in neurons activated independently by the conditioned-stimulus (CS) and the unconditioned-stimulus (US). To achieve that, we used the rAAV2-retro serotype (Tervo et al., 2016) to introduce a CRE-dependent ChR2(H134R)-EYFP (ChR-retro) into the BLA in FDC mice to allow genetic tagging of CS and US-activated ensembles in both local and input neurons (Figure 13).

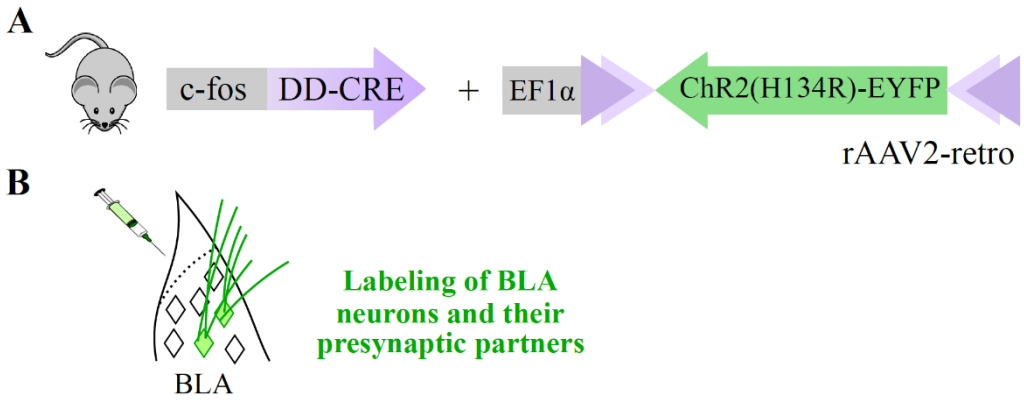


Figure 13. Experimental approach to label BLA neurons and their inputs with channelrhodopsin. (**A**) AAV2-retro carrying a DIO-ChR2(H134R)-eYFP (ChR-retro) construct was bilaterally injected in the BLA of FDC mice, (**B**) leading to expression of ChR-retro in tagged BLA neurons and their presynaptic partners.

The co-injection of an rAAV-DJ containing a CRE-dependent nuclear marker confirmed that the injection was restricted to the BLA (Figure 14). In addition to the BLA, we found significant ChR-retro expression in (from the most intense to the least) the prefrontal cortex (PFC, including prelimbic, infralimbic and mediorbital cortices), the entorhinal cortex (EC,

both lateral and medial portions), the paraventricular nucleus of the thalamus (PVT), the ectorhinal and perirhinal cortices (Ect-PRh), and the insular cortex (InC) (Figure 15). There was inconsistent labeling of the auditory cortex (AuC) when the injection hit more dorsal parts of BLA. Very little to no labeling was found at some known BLA inputs such as the ventral hippocampus (vHPC) (Xu et al., 2016), the medial geniculate nucleus (MGN) and the periaqueductal gray (PAG), suggesting the rAAV2-retro serotype infects cortico-amygdalar inputs more effectively (Figure 16).

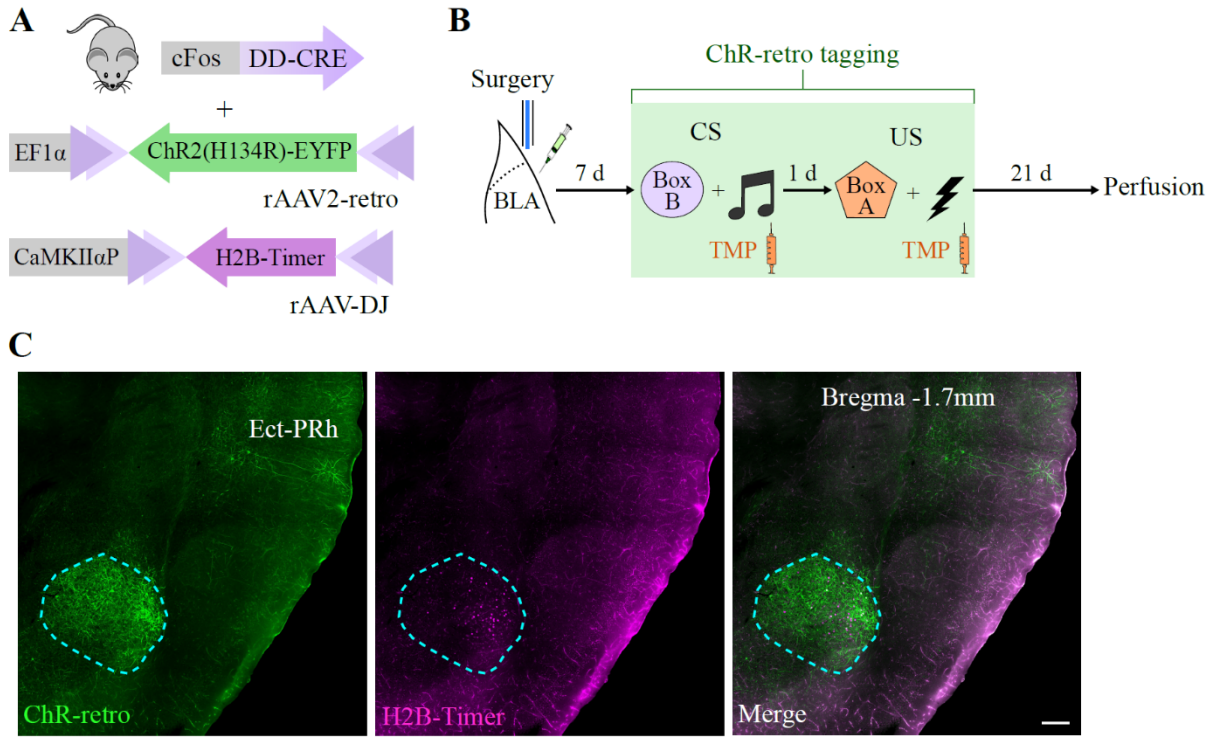


Figure 14. ChR-retro injection was restricted to BLA.
(**A**) rAAV2-retro carrying a DIO-ChR2(H134R)-eYFP (ChR-retro) construct was billaterally co-injected with a non-retrograde rAAV-DJ carrying a H2B-Timer construct in the BLA of FDC mice (**B**) Experimental design to investigate the ChR-retro spread after injection in the BLA. (**C**) Coronal BLA section showing that H2B-Timer expression is restricted to the injection target, while ChR-retro spreads to BLA inputs, such as Ect-PRh. BLA: Basolateral amygdala; Ect-Prh: Ectorhinal and Perirhinal cortices. Scale bar: 200 μm.

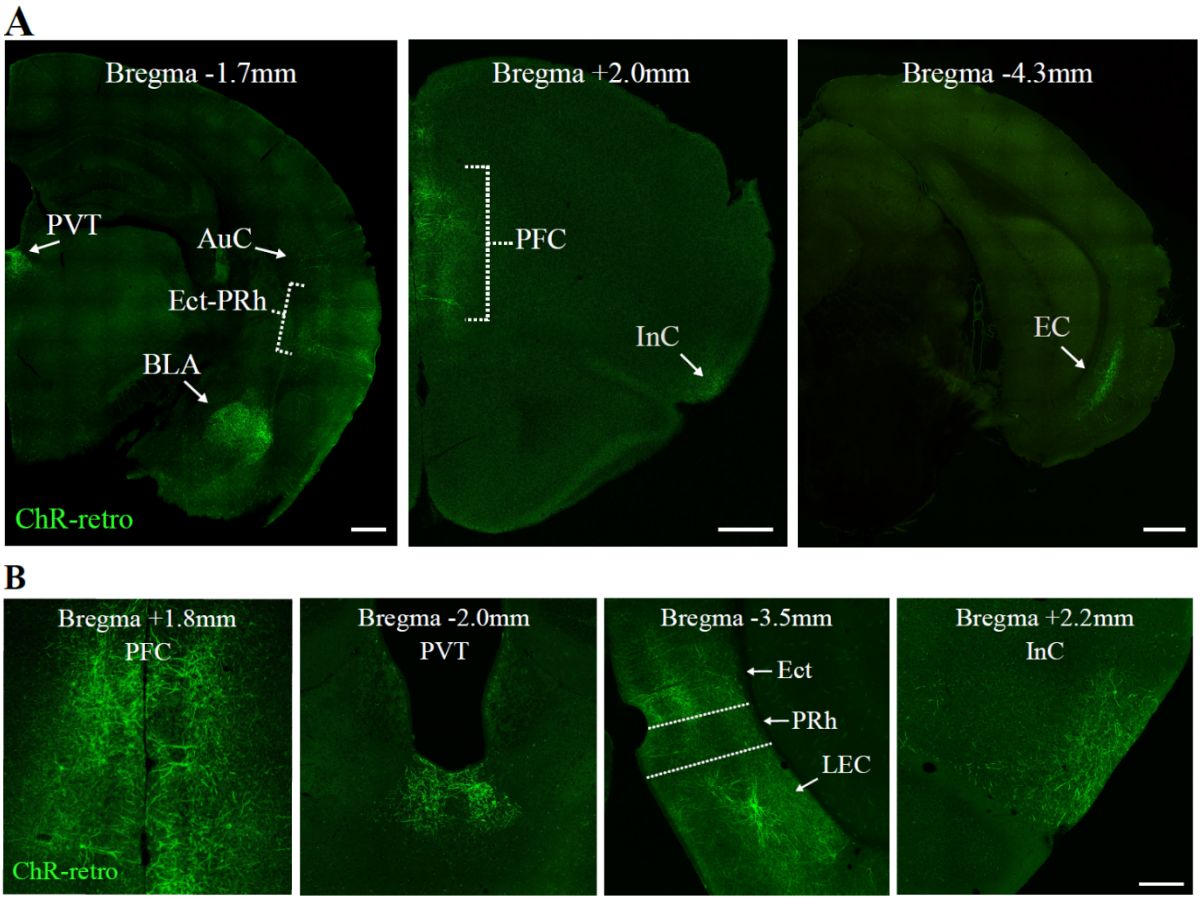


Figure 15. ChR-retro expression pattern after injection in BLA.
(**A**) Coronal sections showing expression pattern of ChR-retro after injection in BLA and tagging during exposure to both CS and US. The brain regions with the most significant expression are labeled. Scale bar: 500 µm. (**B**) Zoomed in coronal sections showing the main areas where ChR-retro expression was detected. Scale bar: 200 µm. AuC: Auditory Cortex (low expression); BLA: Basolateral amygdala; EC: Entorhinal cortex, including lateral and medial regions; Ect-Prh: Ectorhinal and Perirhinal cortices; InC: Insular cortex; LEC: Lateral Entorhinal cortex; MEC: Medial Entorhinal cortex; PFC: Prefrontal cortex, including prelimbic, infralimbic and mediorbital regions;; PVT: Paraventricular nucleus of thalamus, vHPC: ventral hippocampus.

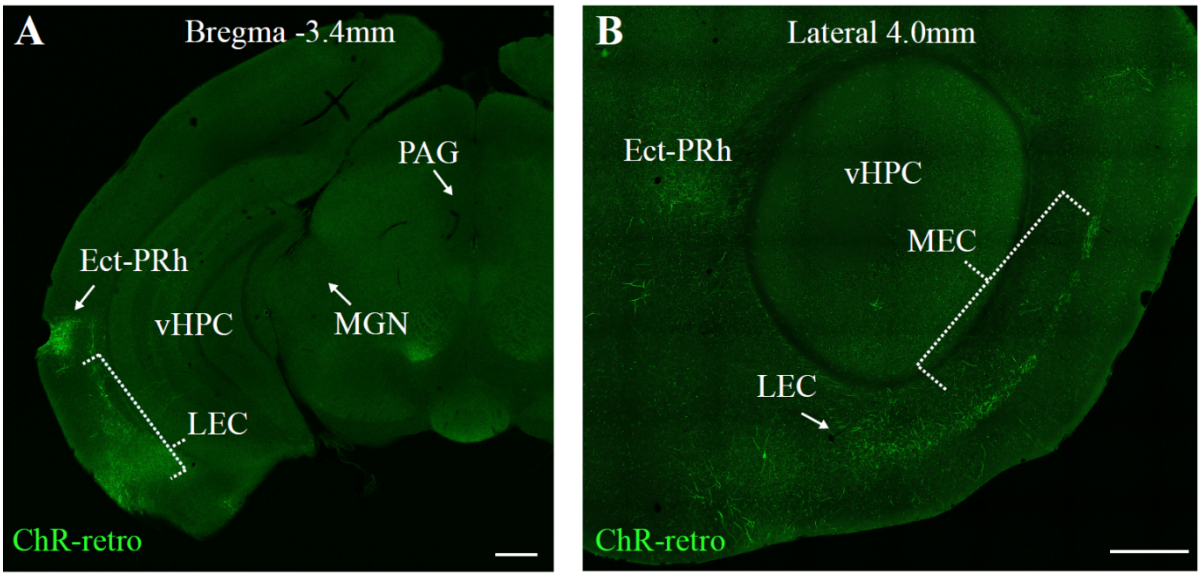


Figure 16. ChR-retro is not expressed in some known inputs to BLA.
(**A**) Coronal section highlighting regions that do not show substantial ChR-retro expression, such as vHPC, MGN and PAG. Scale bar: 500 µm. (**B**) Sagittal section highlighting ChR-retro expression at MEC, as well as LEC and Ect-PRh. Scale bar: 500 µm. BLA: Basolateral amygdala; Ect-PRh: Ectorhinal and Perirhinal cortices; InC: Insular cortex; LEC: Lateral Entorhinal cortex; MEC: Medial Entorhinal cortex; MGN: medial geniculate nucleus; PAG: periaqueductal gray; PFC: Prefrontal cortex, including prelimbic, infralimbic and mediorbital regions; PVT: Paraventricular nucleus of thalamus, vHPC: ventral hippocampus.

In order to test if the potentiation of either CS or US inputs, or both, is sufficient to produce a *de novo* associative fear memory, we used an unpaired training protocol in which the CS (box B + tone) and US (shock) were presented separately on different days to avoid any natural learned association (Figure 17, A and B). To induce synaptic potentiation at BLA inputs, we delivered 100 Hz light pulses through optic fibers implanted bilaterally over the BLA. This stimulation protocol has been shown to induce LTP at auditory inputs to LA (Abdou et al., 2018; Nabavi et al., 2014). The optogenetic stimulation at 100 Hz in the BLA produced a *de novo* associative fear memory to box B only when both CS and US inputs were tagged, while no memory was formed when either one of them was tagged alone (Figure 17C). This artificial memory association was context-specific, as no memory to an untagged box (box C) was

formed. No auditory fear association was formed which may be due to insufficient ChR-retro expression in auditory regions (Figure 15). The optogenetic stimulation did not cause any noticeable performance alterations in anxiety tests, including the elevated plus-maze, the Open field, and the Marble burying tests (Figure 18).

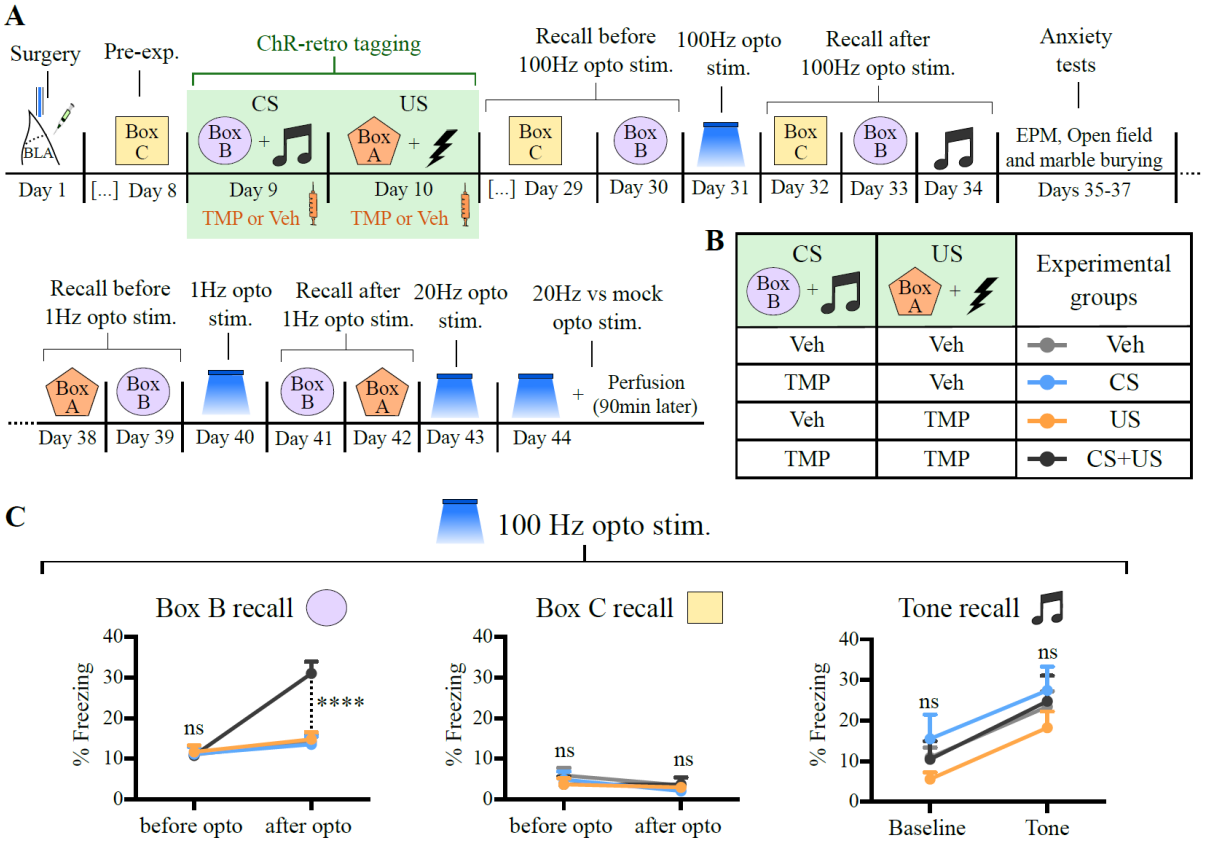


Figure 17. Opto-induced potentiation of both CS+ and US+ tagged neurons in BLA and its pre-synaptic partners is sufficient to create a *de novo* contextual aversive memory.
(**A**) Experimental design for optogenetic-mediated *de novo* memory creation and impairment. (**B**) Experimental groups and their respective tagging regimes, with either TMP or Veh IP injected 20min after each behavior exposure. N = 8 mice per group. (**C**) 100 Hz optogenetic stimulation of BLA neurons and their presynaptic partners generates an artificial memory that is specific to the tagged context (Box B) only when both CS and US are tagged. Opto stim: Optogenetic stimulation. ****P < 0.0001, ns: not significant. Two-way RM ANOVA with Tukey test. Graph bars show mean +/- SEM.

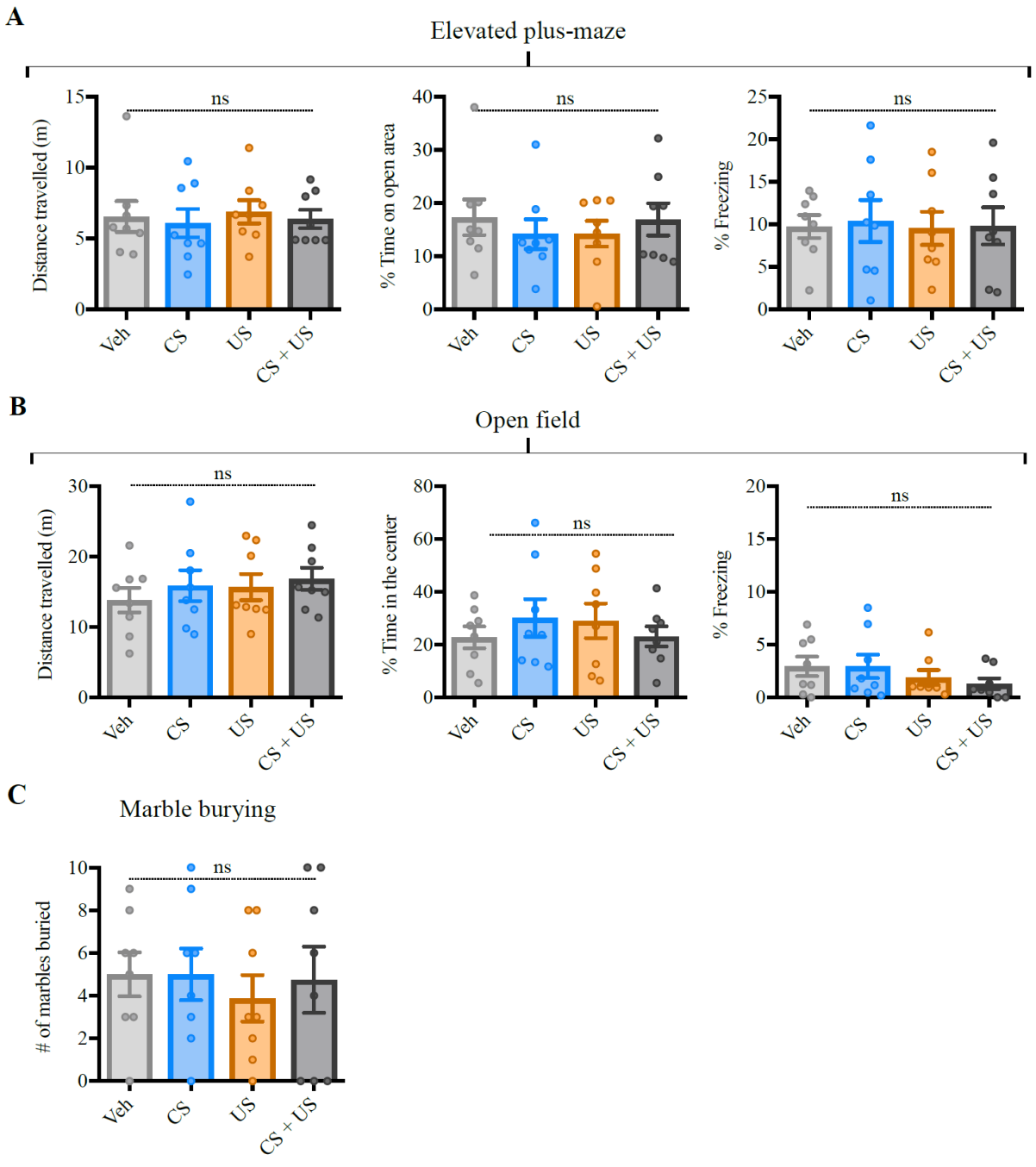


Figure 18. 100 Hz optogenetic stimulation of CS and/or US inputs in the BLA does not interfere with mouse behavior in anxiety tests.

(**A**) and (**B**) No change observed between control (Veh) and other groups on Elevated plus-maze (A) or Open field (B) at distance travelled (left), time on open area/center of the field (center) or freezing (right). (**C**) The number of marbles buried in the Marble burying test were also similar across groups. These tests are part of a large experiment, which is summarized in Figure 17A. Ns, not significant. One-way ANOVA. Graph bars show mean +/- SEM.

Using a double transgenic line that expresses the ChR-variant ChEF in tagged c-fos+ neurons more broadly (Cowansage et al., 2014), we found that the same optogenetic stimulation protocol at 100 Hz produces a *de novo* fear association to both the tone and context (Figure 19).

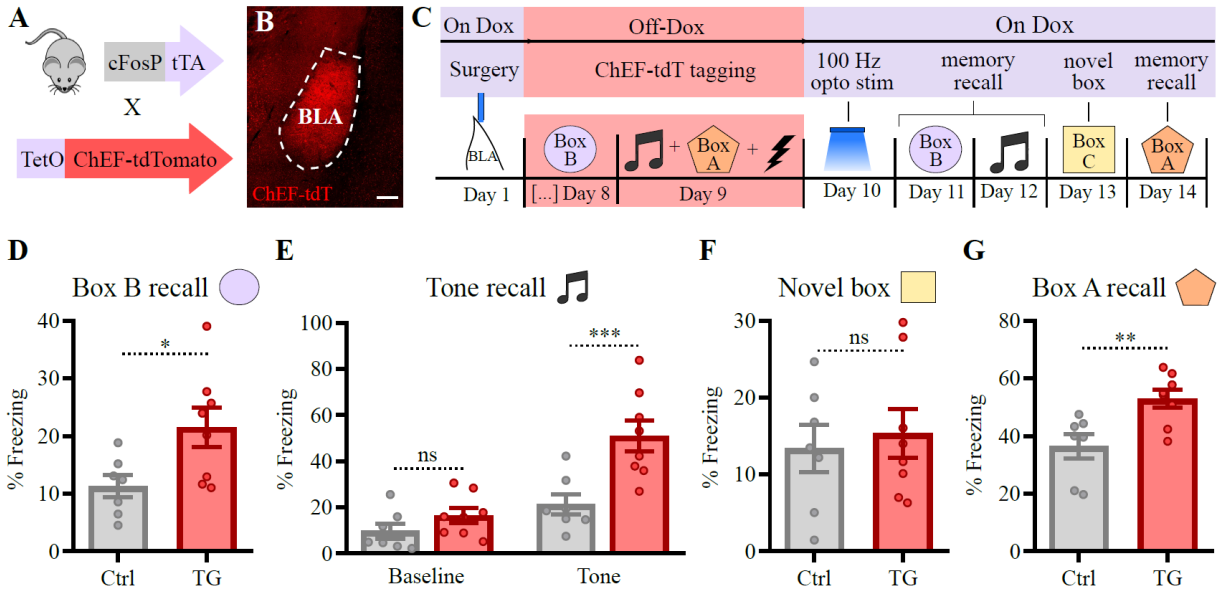
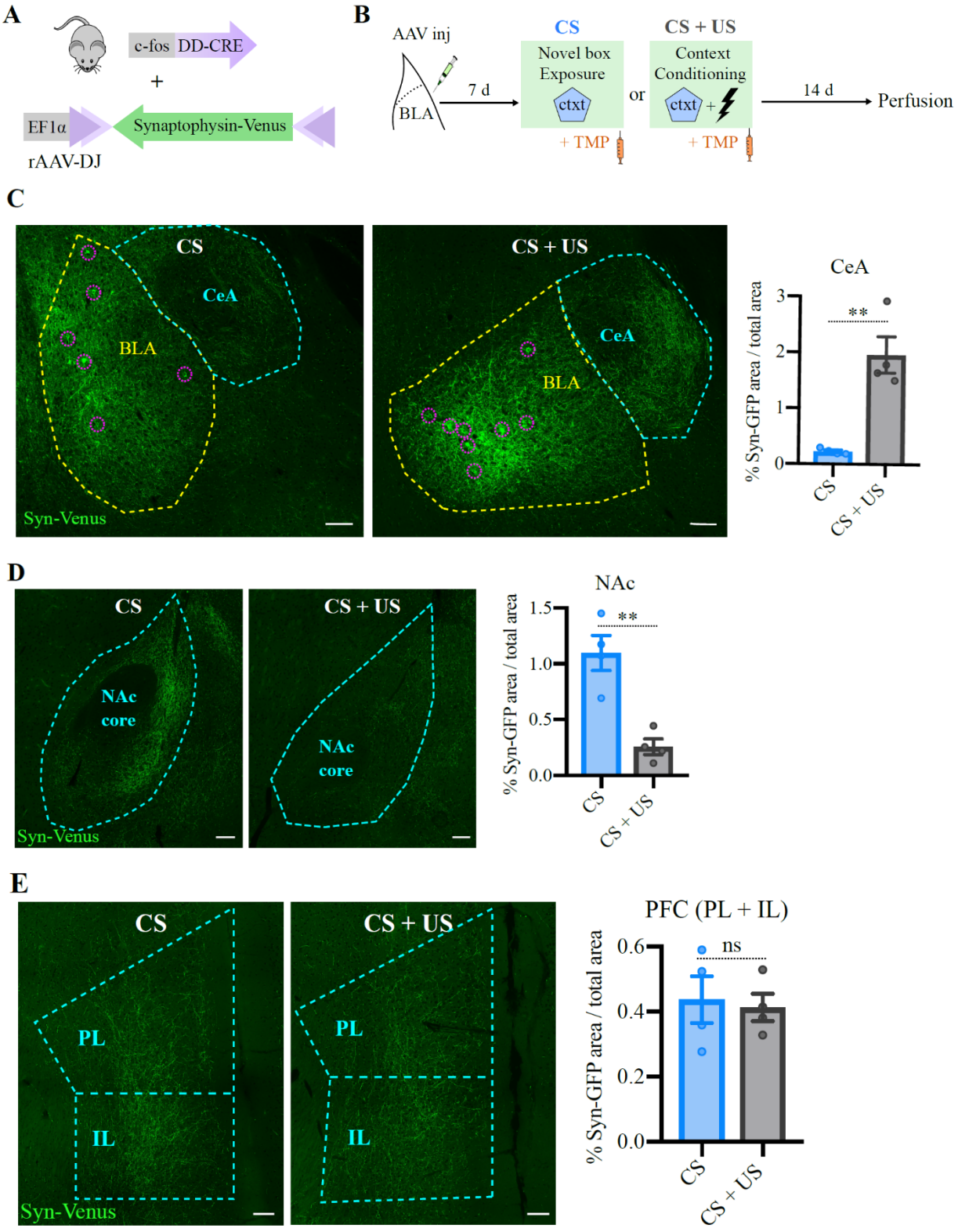


Figure 19. 100 Hz optogenetic stimulation of CS and US inputs in the BLA creates *de novo* aversive associative memory in transgenic mouse expressing ChEF (a ChR variant) in tagged c-fos+ neurons.

(**A**) Double transgenic mouse expressing ChEF fused to tdTomato (tdT) under the control of the Tet system driven by the c-fos promoter. (**B**) Coronal section at Bregma -1.2 mm showing ChEF-tdT expression in the BLA one day after tagging shown in (C). Scale bar: 200 µm. (**C**) Experimental design used to test whether 100 Hz optogenetic stimulation of CS and US inputs in the BLA may create a *de novo* memory association. (**D**) to (**G**) 100 Hz light stimulation induced a *de novo* fear memory to box B (D) and to the tone (E) (unpaired with the shock during tagging). Effect was context specific, as no enhanced freezing to untagged box was detected (F). The natural fear memory to box A was potentiated by 100 Hz light stimulation (G). Control group (Ctrl) was composed of single transgenic mice from the same cohort of the double transgenic group (TG). N = 7-8 mice per group. ***P < 0.0001 **P < 0.01, *P < 0.05, ns, not significant, unpaired t test [(D), (F) and (G)], or two-way RM ANOVA with Bonferroni test (E). Graph bars show mean +/- SEM.

One model of fear learning posits that it occurs through the potentiation of CS inputs onto amygdala neurons (Blair et al., 2001), suggesting that it would be sufficient to generate a *de novo* contextual fear memory association, which did not occur in our hands (Figure 17, "CS" group, in blue). One possible explanation is that the indiscriminate potentiation of CS inputs might result in no clear behavioral outcome (Nabavi et al., 2014), as BLA is a critical component of behaviors of distinct emotional valence (Namburi et al., 2016). Indeed, BLA neurons tagged during box exposure (CS) project less heavily to the central amygdala (CeA), a known fear center, and more heavily to the nucleus accumbens (NAc) core than BLA neurons tagged during context conditioning (CS + US) (Figure 20), which suggests that different neuronal populations are activated by the CS alone compared to CS + US (Grewe et al., 2017). In our setup, the tagging of US neurons might be crucial to direct the optogenetic manipulation towards aversive behaviors.

Figure 20. The projection targets of BLA neurons that activated by the CS are different from the ones that are activated by CS + US.
(**A**) rAAV-DJ encoding a DIO-Synaptophysin-Venus (Syn-Venus) was unilaterally in the BLA of FDC mice. (**B**) Experimental design to compare the projection targets between BLA neurons activated by CS (novel box) and CS + US (context conditioning). (**C**) Representative coronal sections showing the injection site at BLA (yellow dashed line), with visible targeted neurons highlighted in purple dashed circles, and the projections to the central amygdala (CeA), stronger in the CS + US group (center), quantified in the graph on the right. (**D**) Representative coronal Nucleus Accumbens (NAc) sections showed the opposite trend, with stronger projections in the CS group, quantified in the graph on the right. (**E**) Representative coronal pre-frontal cortex sections, including prelimibic (PL) and infralimbic (IL) regions, where no substantial difference between the groups was found (graph on the right). Scale bar: 100 μm. N = 4 mice per group. **P < 0.01, ns, not significant, unpaired t test. Graph bars show mean +/- SEM.


A
c-fos DD-CRE
+
EF1α
Synaptophysin-Venus
rAAV-DJ
B
AAV inj
BLA
7 d
CS
Novel box Exposure
ctxt
+ TMP
or
CS + US
Context Conditioning
ctxt
+ TMP
14 d
Perfusion
C
CS
CeA
BLA
Syn-Venus
CS + US
CeA
BLA
CeA
% Syn-GFP area / total area
**
CS
CS + US
D
CS
NAc core
Syn-Venus
CS + US
NAc core
NAc
% Syn-GFP area / total area
**
CS
CS + US
E
CS
PL
IL
Syn-Venus
CS + US
PL
IL
PFC (PL + IL)
% Syn-GFP area / total area
ns
CS
CS + US

To test whether we could reverse the artificial memory association to box B, we used 1 Hz optogenetic stimulation (Figure 17A), as it has been shown to induce LTD in auditory inputs to LA (Kim and Cho, 2017; Nabavi et al., 2014). We found that, after 1 Hz light pulses, the freezing levels to box B returned to baseline, suggesting reversal of the artificial context memory (Figure 21). Interestingly, the memory to box A (tagged with the US, Figure 17A) was not significantly affected, which suggests that the natural recall may also occur through routes that were insufficiently tagged by ChR-retro, including vHPC (Xu et al., 2016) and subcortical regions (Figure 16). Despite the need for tagging both CS and US inputs for *de novo* memory generation, we find that the tagging of the US neurons alone is sufficient to lead to light-induced freezing during optogenetic stimulation at 20 Hz in a novel box (Figure 21). This stimulation protocol induces enhanced c-fos expression in BLA, which overlaps with the US-tagged cells (Figure 22).

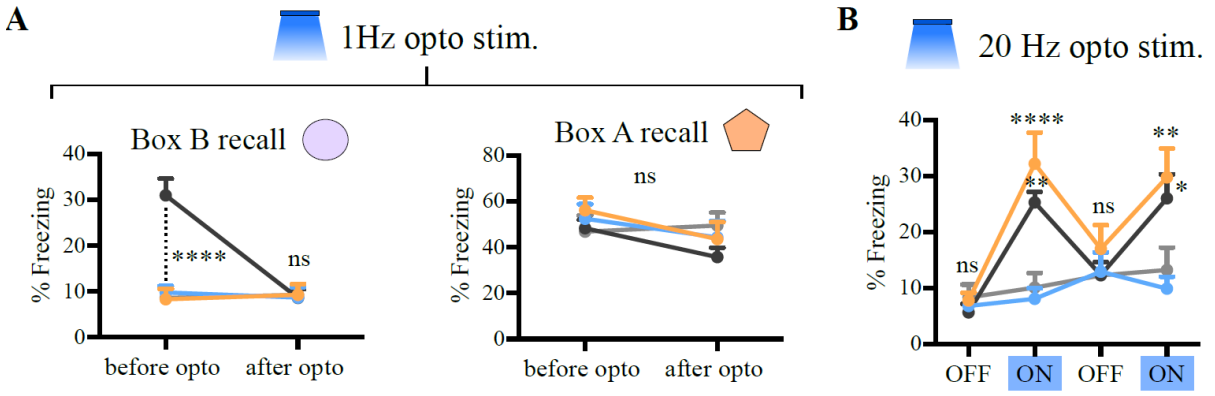


Figure 21. 1 Hz optogenetic stimulation disrupts artificial memory.
(**A**) 1 Hz optogenetic stimulation reverses the artificially created memory to box B, but not the natural memory to box A. (**B**) Freezing behavior during 20 Hz light stimulation occurs only when the US is tagged. Opto stim: Optogenetic stimulation. ****P < 0.0001, **P < 0.01;,* P< 0.05, ns: not significant. Two-way RM ANOVA with Tukey test. Graph bars show mean +/- SEM.

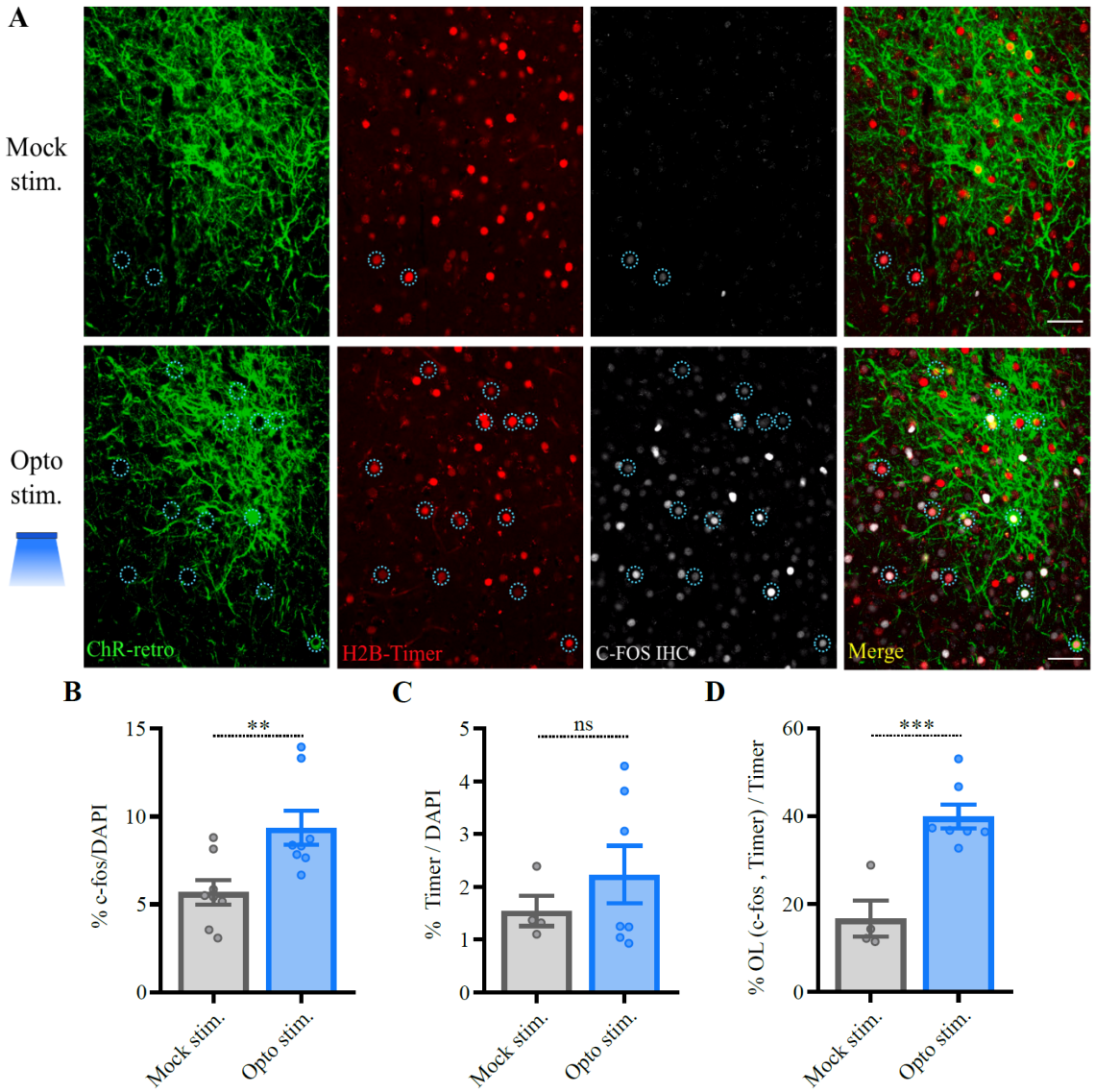


Figure 22. 20 Hz optogenetic stimulation induces increased c-fos levels in the BLA, which overlaps with tagged US neurons.

(**A**) Representative coronal BLA section showing the expression of ChR-retro and the co-injected nuclear marker H2B-Timer along with c-fos immunohistochemistry (IHC) in animals perfused 90min after 20Hz opto stim. or mock stim. Dotted circles highlight tagged cells that were reactivated (both Timer+ and c-fos+, quantified in D). The complete experimental design is illustrated in Figure 3C. Only animals that were tagged during US exposure, therefore exhibited freezing to 20 Hz opto stim. (Figure 21) were included in this experiment ("US" and "CS + US" were equally divided among the two groups and pooled together, as there was no difference between them). Scale bar: 50 µm. (**B**) 20 Hz Opto stim. induced increase in overall c-fos levels in BLA. N = 8 mice per group. (**C**) Timer levels were similar between groups. (**D**) Neurons that were tagged during US exposure (Timer+) tend to be reactivated (c-fos+) during 20 Hz opto stim. N = 4 in "mock stim." group and 8 in "opto stim." group in (C) and (D). ***P < 0.0001 **P < 0.01, ns, not significant, unpaired t test. Graph bars show mean +/- SEM.

One caveat of optogenetic stimulation is possible off-target effects both upstream and downstream of the target area due to the stimulation-driven circuit response (Otchy et al., 2015). To circumvent this limitation, we developed a strategy to biochemically induce LTP only in the BLA with the expression of the triple mutant CamKIIα T286D-T305A-T306A (CK2-DAA), which has been shown to induce LTP in hippocampal organotypic slices (H. J. Pi et al., 2010). Using a similar approach to that described in Figure 6, we bilaterally injected an AAV containing a CRE-dependent construct expressing CK2-DAA into the BLA of FDC mice (Figure 23A). To test whether CK2-DAA could induce LTP in BLA neurons in the absence of shock exposure, we tagged neurons during a novel box exposure, and 7d later, prepared slices for whole-cell recordings (Figure 23B). Indeed, the CK2-DAA+ (GFP+) neurons exhibited an increase in AMPAR/NMDAR ratio when compared to the neighboring GFP- neurons (Figure 23C), without affecting their intrinsic excitability (Table 2). This increase in AMPAR/NMDAR is not simply due to overexpression of the wild-type CaMKIIα (WT-CK2) (Figure 23C).

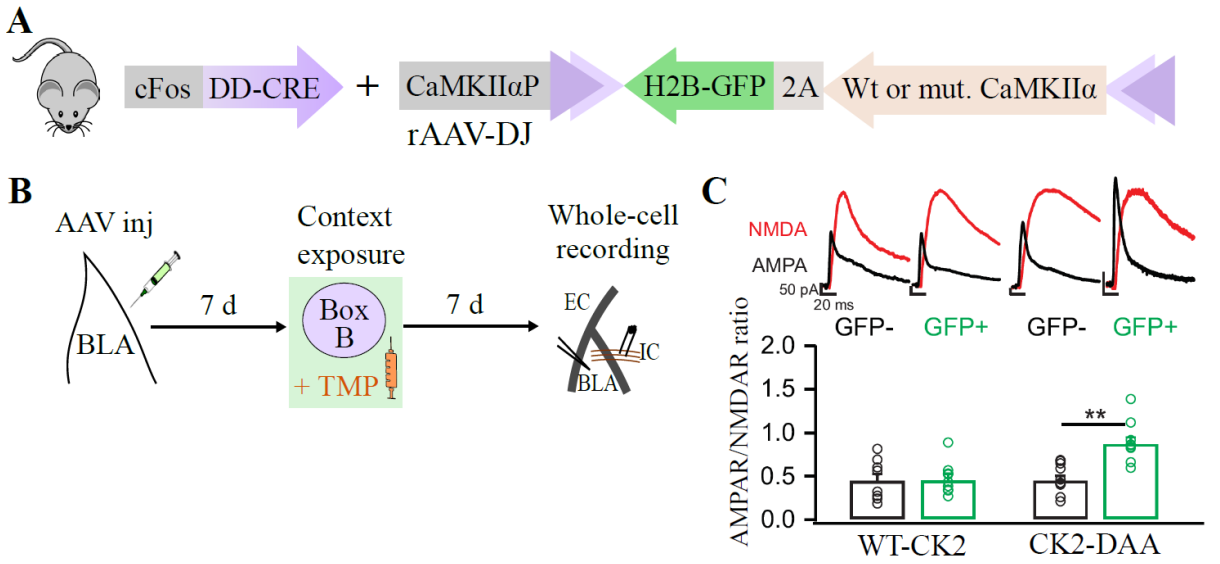
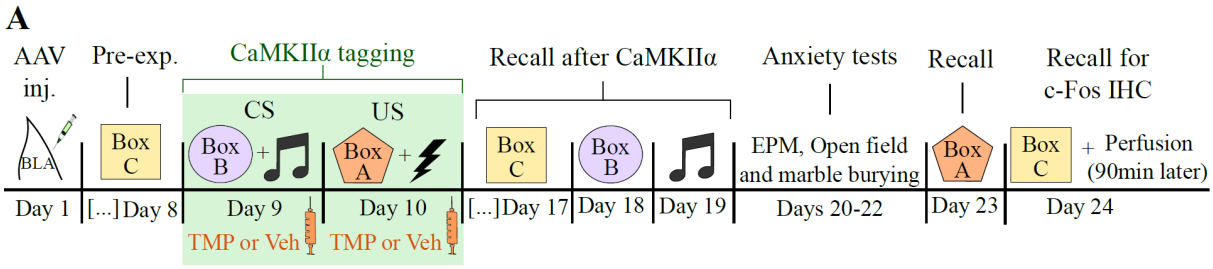


Figure 23. CamKIIα T286D-T305A-T306A (CK2-DAA) expression in BLA neurons induces increase in AMPAR/NMDAR ratio in the absence of associative training.
(**A**) rAAV-DJ carrying a DIO-CK2-2A-H2BGFP construct was injected bilaterally in the BLA of FDC mice. (**B**) Experimental design to test whether CK2-DAA induces LTP in the BLA in the absence of US exposure. (**C**) CK2-DAA expression induces increase in AMPAR/NMDAR ratio, whereas wild-type CaMKIIα (WT-CK2) overexpression does not affect it. **P < 0.01; unpaired t-test. Graphs show mean +/- SEM.

Table 2. CaMKIIα-T286D/T305A/T306A (CK2-DAA) expression in c-fos+ BLA neurons tagged during novel box exposure does not affect the intrinsic excitability of these neurons.
Results of unpaired t tests and the respective P value is included for every corresponding measure. AP: action potential, V resting: resting potential, Rm: Membrane resistance, AHP: afterhyperpolarization.

	CK2-DAA-	CK2-DAA+	t	p
	n= 15	*n = 9*		
AP threshold (mV)	-47.1 ± 1.3	-47.0 ± 2.1	0.06	0.95
AP amplitude (mV)	73.9 ± 2.9	79.2 ± 1.4	1.28	0.21
AP half width (ms)	0.82 ± 0.04	0.87 ± 0.06	0.77	0.45
V resting (mV)	-78.9 ± 1.8	-74.5 ± 2.0	1.55	0.14
Rm (Mohm)	167.9 ± 12.9	185.9 ± 36.8	0.55	0.59
AP numbers at 300pA	3.2 ± 0.3	3.7 ± 0.5	0.84	0.41
Latency to spike (ms)	6.5 ± 0.8	7.7 ± 1.6	0.75	0.46
	n= 9	*n = 7*		
AHP (mV)	7.1 ± 0.5	5.8 ± 0.8	1.33	0.21

If plasticity in BLA alone is sufficient to create context fear associations, which neurons and synapses need to be potentiated? To address that question, we used a similar unpaired training protocol to the one described in Figure 17, in which CS and US were tagged independently on different days (Figure 24). We found that the potentiation of BLA neurons tagged during US exposure alone is sufficient to drive both tone and context-mediated aversive associations, and that this is generalized to multiple contexts. (Figure 25). Despite enhanced cue induced freezing levels, no significant changes in locomotion or common anxiety behaviors were observed during elevated plus-maze, open field or marble burying tests (Figure 26).



B

CS (Box B + ♪)	US (Box A + ⚡)	Experimental groups
Veh	Veh	Veh
TMP	Veh	CS
Veh	TMP	WT-CK2
Veh	TMP	ON Hab
Veh	TMP	US
TMP	TMP	CS + US

Figure 24. Experimental design to test whether the induction of synaptic potentiation in BLA neurons is sufficient to create a *de novo* memory.
(**A)** Experimental design to test whether CK2-DAA mediated-potentiation creates a *de novo* memory association. (**B**) Experimental groups and their respective tagging regimes, with either TMP or Veh IP injected 20 min after each behavior exposure. Mice in the ON hab (overnight habiuation) group were kept overnight in box A before shock exposure to prevent any conditioning (Figure 1C).

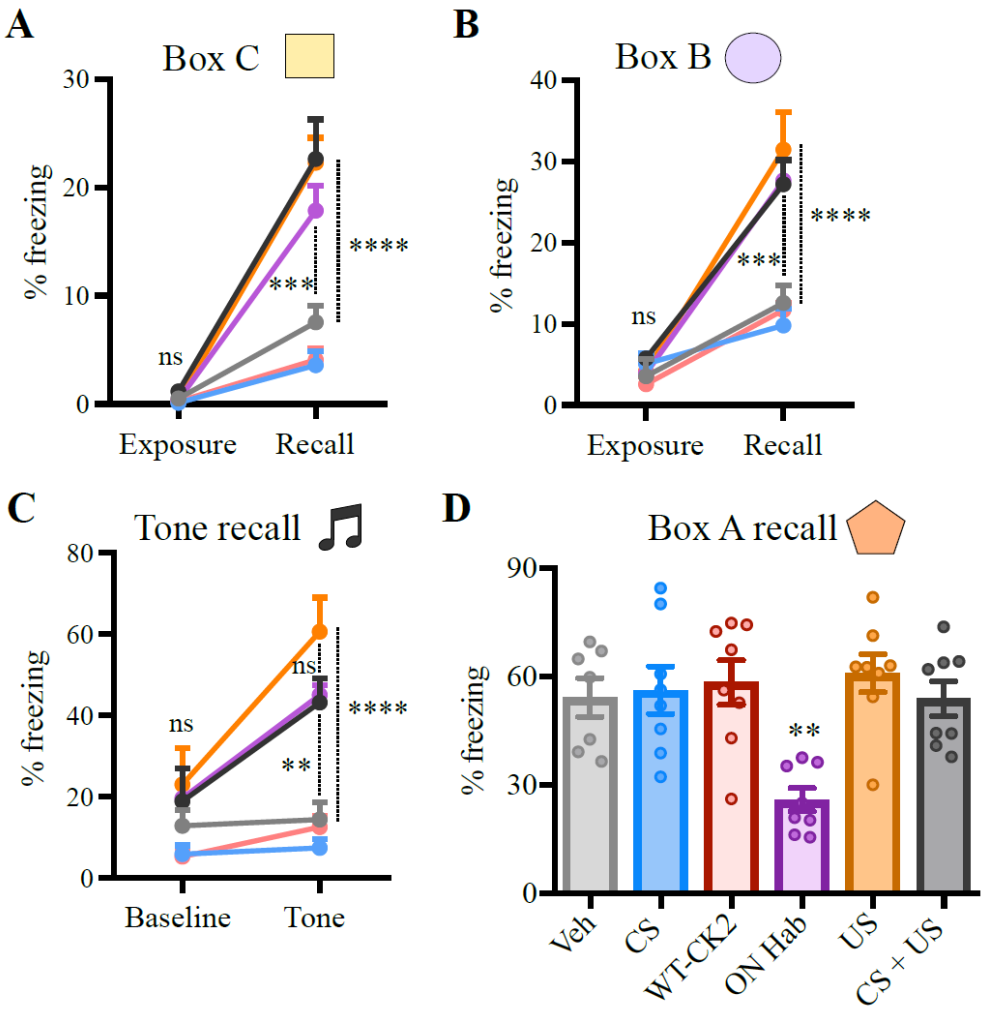


Figure 25. CaMKIIα-T286D/T305A/T306A (CK2-DAA) expression in US-responsive BLA neurons leads to generalized fear association.

(**A**) to (**C**) CK2-DAA potentiation of US neurons led to indiscriminate fear associations, including the untagged box C (A). (**D**) The ON Hab group showed reduced freezing levels to box A. Experimental design detailed in Figure 24. ON Hab = overnight habituation group. N = 8 mice per group, except in the Veh group, where N = 7. **P < 0.01, ***P < 0.001, ****P < 0.0001, ns, not significant; two-way RM ANOVA with Tukey test [(A) to (C)] or one-way ANOVA with Tukey test (D). Graphs show mean +/- SEM.

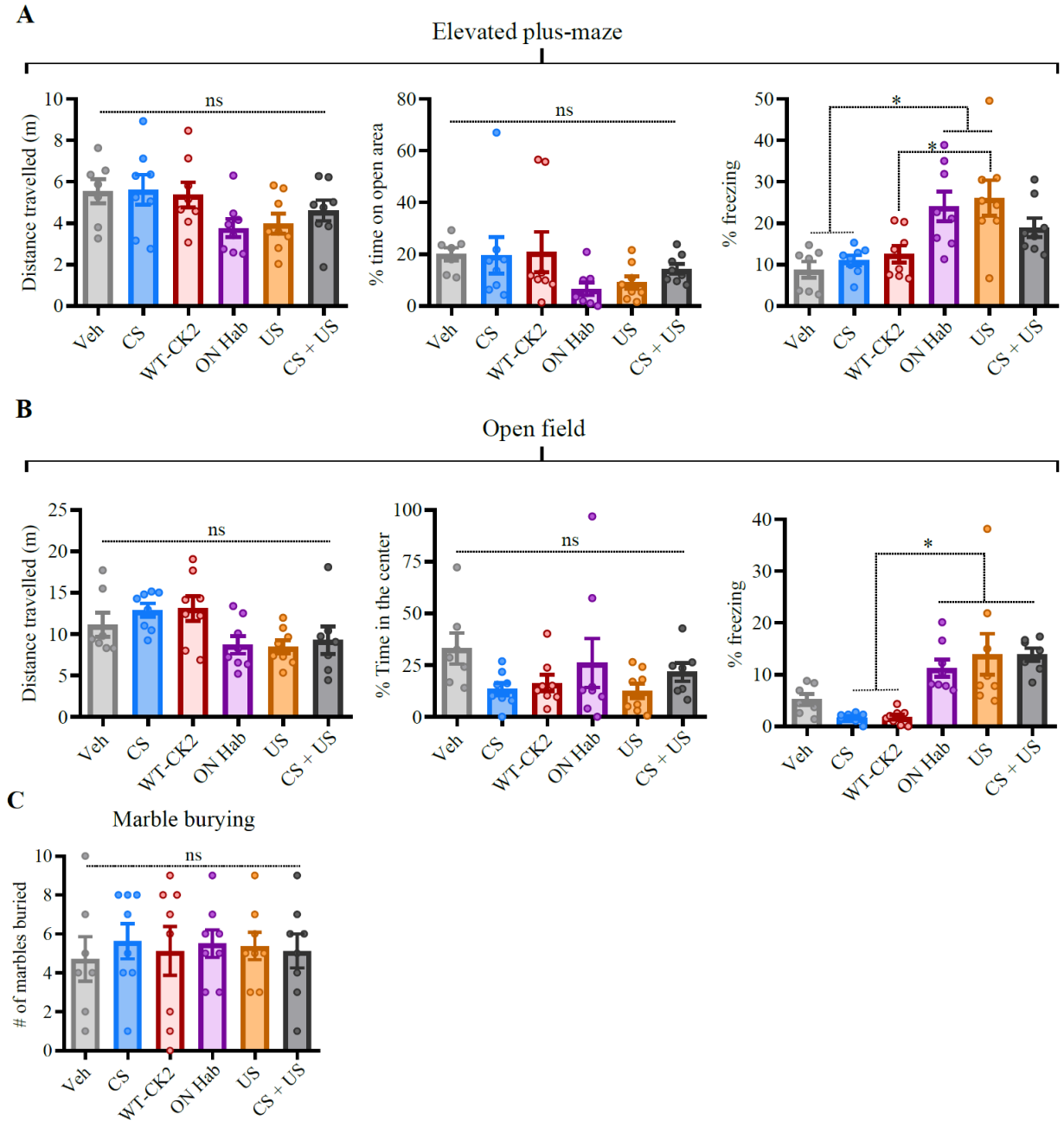


Figure 26. CK2-DAA expression in the BLA does not interfere with locomotion or behavior in anxiety tests, despite affecting freezing.

(**A**) and (**B**) No change observed between control (Veh) and other groups on Elevated plus-maze (A) or Open field (B) at distance travelled (left) or time on open area/center of the field (center), despite some difference in freezing levels (right). (**C**) The number of marbles buried in the Marble burying test were also similar across groups. The tests are part of a large experiment, which is summarized in Figure 24. N = 8 mice per group, except in the Veh group, where N = 7, and on open field, where 1 outlier animal was excluded from "CS + US group" due to elevated freezing at the center of the arena. *P < 0.05, ns, not significant. One-way ANOVA with Tukey test. Graph bars show mean +/- SEM.

To rule out the possibility that any new fear memories were due to the formation of hybrid associations with the natural memory to box A (Garner et al., 2012), we included a control in which the animals were habituated overnight to box A (ON Hab) before the exposure to the shock to prevent any conditioning (Impey et al., 1998) (Figure 1C). Still, we observed generalized context fear associations (Figure 25, purple line), even though the freezing to box A was reduced to a level similar to untrained boxes B and C (Figure 25D). Staining for c-fos induced during exposure to box C showed that CK2-DAA expression promotes the reactivation of neurons that were tagged during US exposure (GFP+) relative to neurons tagged with a WT-CK2 control (Figure 27). This result suggests that the activation of US neurons facilitated by CK2-DAA induced-synaptic potentiation drives generalized context induced fear.

Overall, our results indicate that synaptic potentiation of CS inputs onto US BLA neurons is a necessary and sufficient form of plasticity that underlies context fear conditioning, fulfilling all three criteria for the identification of an engram, as outlined in Chapter 1.

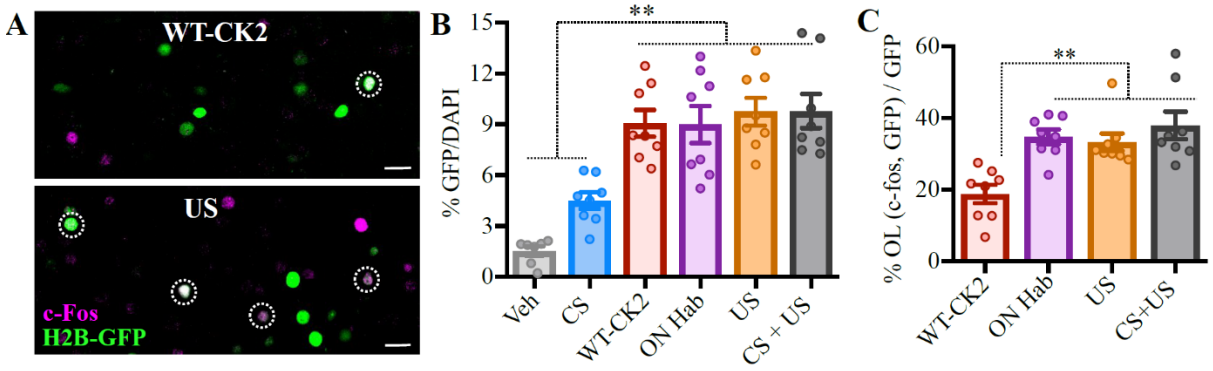


Figure 27. CK2-DAA expression promotes the reactivation of neuron tagged during US exposure. (**A**) Representative coronal BLA sections showing overlap between activity markers during exposure (H2B-GFP) and recall to box C (c-fos IHC). Scale bar: 20 µm. (**B**) The percentage of tagged cells (H2B-GFP+) were smaller in Veh and CS groups. (**C**) CK2-DAA expression promotes reactivation of neurons tagged during US exposure. OL: overlap. **P < 0.01; one-way ANOVA with Tukey test. Graphs show mean +/- SEM.

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Chapter 3: Discussion

The dangerous man is the one who has only one idea, because then he'll fight and die for it. The way real science goes is that you come up with lots of ideas, and most of them will be wrong.

Francis Crick
New Yorker, 2011

Our study is the first, to our knowledge, to show synaptic potentiation in BLA neurons after context conditioning, and to demonstrate that this plasticity is both necessary and sufficient for memory recall. Another recent study reported an increase in synaptic strength in LA after contextual conditioning (Butler et al., 2018), although the authors do not properly control for the possibility of CS or US-only mediated plasticity. Interestingly, in many of the studies examining learning-induced plasticity in the LA/BLA, the behavioral training would be expected to produce a contextual fear memory in addition to tone conditioning, yet the synaptic changes observed were only dependent on tone-shock pairing contingencies (McKernan and Shinnick-Gallagher, 1997; Namburi et al., 2015; Tsvetkov et al., 2002). This discrepancy might be explained by the fact that our recordings targeted neurons activated during training (c-fos+), while previous studies have recorded from randomly targeted neurons, which suggest that a context representation in BLA is sparser than an auditory representation. Alternatively, the discrepancy might derive from the amygdala subregion being targeted: our recordings were concentrated in the anterior BLA [Bregma -0.8 mm to -1.5 mm; (Paxinos and Franklin, 2012)], while other studies targeted the LA (McKernan and Shinnick-Gallagher, 1997; Tsvetkov et al., 2002) or LA/BLA (Namburi et al., 2015).

A previous study showed evidence for presynaptic potentiation in inputs to BLA after contextual conditioning, with an increase in the miniature EPSC frequency, but not amplitude, and a decrease in the paired-pulse ratio (Nonaka et al., 2014), which diverge from our findings of absence of presynaptic potentiation following fear conditioning (Figure 3). This divergence might be explained by the fact that our recordings were based on stimulation of internal capsule inputs, while Nonaka and colleagues reported that the presynaptic potentiation was specific to external capsule inputs. Another important difference between this and our study is the age of the mice used for recordings: we used 12-20 weeks old animals, while they used much younger animals, 3-5 weeks old. Still, this study points out to another type of plasticity following contextual fear conditioning. However, considering the experimental design, the possibility of CS-only mediated potentiation (context encoding) cannot be ruled out.

We have also demonstrated that context extinction reverses synaptic potentiation in learning-induced BLA neurons, while re-training reinstates the plasticity in the same set of neurons (Figure 5). While the literature on context extinction is scarce, studies on tone extinction after auditory fear conditioning suggest that extinction is a new form of learning, as it is context dependent and the return of the extinguished behavior is common after some time (Dunsmoor et al., 2015; Maren et al., 2013). Moreover, different regions of the prefrontal cortex seem to be involved with either recall and extinction in tone conditioning (Giustino and Maren, 2015). Indeed, a recent study looking at specific auditory inputs to the lateral amygdala found that the synaptic potentiation following tone fear conditioning was not affected by tone extinction (Kim and Cho, 2017b), which contrasts with our findings [although at least one study reported depotentiation after tone extinction (Kim et al., 2007)]. While extinction to the tone typically involves putting the animal in a different context from the conditioning box, context

extinction is carried out by putting the animals back into the same conditioning context, providing all the cues the animal had during the conditioning except for the US. Perhaps the greater similarity with the training experience during context extinction favors memory updating, leading to reversal of learning-induced potentiation, while the presence of a single cue during tone extinction in a completely different environment leads to the formation of a specific new extinction memory. It would be interesting to compare the effect on learning-induced plasticity of tone extinction conducted in the conditioning box (or similar boxes) versus different boxes. This could, for instance, inform exposure-based therapies to post-traumatic stress disorder (PTSD) patients about the potential importance of context similarity with the traumatic event.

Using the FDC mouse, we have demonstrated that reversing synaptic potentiation in learning-induced BLA neurons impairs memory recall (Figure 8). We achieved that by expressing the LTD-inducing CamKIIα-T286D (CK2-D) in these neurons. Interestingly, CK2-D was first thought to be constitutively-active, therefore LTP-inducing (Bejar et al., 2002; Mayford et al., 1996) until more recently when recording experiments demonstrated it actually induces LTD by autophosphorylation of an inactivating site (Hyun Jae Pi et al., 2010). Despite being present during memory recall, CK2-D expression did not affect the intrinsic excitability of tagged neurons (Figure 11 and Table 1), in contrast to what was previously reported in striatal neurons, when CK2-D was transgenically expressed throughout development (Kourrich et al., 2012). Indeed, previous work conducted in a non-cell specific manner demonstrated that transient expression of CK2-D after training and before recall is sufficient to induce memory impairment (Rossetti et al., 2017; Yasuda and Mayford, 2006).

It is intriguing that our 1 Hz optogenetic stimulation did not affect the natural memory to box A, which was paired with the foot shocks, despite impairing the artificially generated memory to box B (Figure 21). As we pointed out, this may be due to insufficient ChR labeling of brain regions known to project to the BLA, such as vHPC (Xu et al., 2016) and subcortical regions (Figure 16), which might support the recall of a natural memory. Alternatively, the fact that the natural memory is substantially older than the artificially generated one (30 days vs 9 days) might explain the disparity, as the role of BLA in memory recall might reduce as time goes by (Do Monte et al., 2016). A third possibility is that the natural memory achieved a higher level of synaptic potentiation after 100 Hz optogenetic stimulation, much higher than the artificially induced association, and that the 1 Hz light stimulation protocol used here was not sufficient to weaken the natural memory.

Together, our results show that the essential underlying cellular plasticity for forming context fear associations is the potentiation of CS inputs onto US responsive neurons in the BLA that in turn project selectively to the central amygdala and the fear output circuit (Tovote et al., 2015). This suggests that even for hippocampal dependent tasks such as context recognition, which involve the integration of multimodal sensory cues, the fear association takes place in the amygdala and the specificity of the response is likely determined by the nature of the inputs activated during learning and retrieval. In fear conditioning it is the aversive shock that is "recalled", as the animals are actually present in the context box during a retrieval trial. According to this view, the role of hippocampal and cortical structures is to respond to the context cues to recruit the same neuronal ensembles as in the original context exposure during learning, with fear being expressed by the increased synaptic plasticity of BLA inputs and subsequent activation of the US linked fear circuit. Previous lesion and pharmacological

manipulations of various hippocampal and cortical structures have been shown to reduce conditioned fear (Corcoran et al., 2011; Frankland et al., 2004; Gale et al., 2004; Kim and Fanselow, 1992; Lesburguères et al., 2011; Phillips and LeDoux, 1992; Quinn et al., 2005). This may reflect altered processing of contextual information, preventing reactivation of the appropriate BLA inputs during retrieval, rather than a direct requirement for plasticity in these areas. As the animals are already in the box during memory recall, plasticity involved in context encoding may only play an auxiliary role in context conditioning. Conversely, context encoding-related plasticity might be critical in more complex tasks involving context discrimination or maze navigation.

Our results contribute to the debate regarding the distributive vs localized nature of memory. We showed that synaptic potentiation of US-responding BLA neuronal ensembles is sufficient to induce an aversive memory response. This suggests that, at least for the CS-US association, memory is stored by only a few neurons in a single brain region. It is important to note, however, that the magnitude of the behavior response was about 50% of the natural memory, suggesting that other sites of plasticity in BLA or elsewhere in the fear circuit might contribute to modulate the magnitude of the response. Moreover, while simple learning associations might be stored in a given brain region, more complex memories likely require plasticity in neurons distributed more widely throughout the circuit.

3.1. Potential Caveats

A general caveat in the study of memory in animal models is that, unfortunately, animals don't verbally answer our questions, so we have no way to know what the animal is thinking or

remembering. Using the behavioral output as a proxy for memory recall, we controlled for alternative explanations, such as a pure (lack of) motor response, but we have no way to verify the impact of our manipulations on the quality of memories or their meaning, which have a critical importance for us as humans. This is an important caveat to keep in mind, which we can only diminish by comparing our study with similar studies conducted in humans. Indeed, studies involving human subjects with amygdala damage indicate that a critical function of the human amygdala is to enhance the perception of stimuli that have emotional significance (Adolphs, 2008; Anderson and Phelps, 2001), suggesting that in our manipulation of synaptic plasticity in the mouse BLA, we might have altered the emotional response of the mouse to different stimuli.

Another potential caveat of our study concerns the methodology, which relies on c-fos expressing neurons. Although c-fos expression has been linked to neuronal spiking and increased levels of intracellular calcium (Schoenenberger et al., 2009; Sheng et al., 1993), and we have used a transgenic mouse with a knock-in into the c-fos locus (which reduces the likelihood of aberrant gene expression) and perhaps the tightest tagging window currently available (Dillingham et al., 2019), it is unclear what defines a c-fos+. Moreover, the parameters for cfos induction may differ among brain regions, which could impact the conclusions obtained by this and other current studies using similar technologies. For instance, c-fos+ neurons in the hippocampus seem to represent a specific population of place cells that are highly context specific and demonstrate preferential remapping of their place fields (Tanaka et al., 2018). Whether BLA cfos+ neurons reflect aspects of neural activity other than simple spiking rates, as hippocampal neurons appear to, is unclear. Over the years, with the advent of new technologies, the nature of c-fos+ and other IEGs ensembles, their role in learning and

memory, and how they compare to the theoretical cell assemblies define by Hebb (Hebb, 1949) should become more clear.

Due to the limitations of current technology, our optogenetic manipulations lack the temporal coordination and order that a natural stimulus might follow, which could lead to unpredictable circuit consequences (Otchy et al., 2015). This was one of the reasons why we used an alternative method, involving the triple mutant CamKIIα-T286D-T305A-T306A (CK2-DAA), to demonstrate that synaptic potentiation of BLA inputs onto US neurons is sufficient to instantiate a memory association.

Another potential caveat of our study is that we did not show the effect of optogenetic stimulation on synaptic potentiation. We relied on previous studies that have shown that 100 Hz optogenetic stimulation induces LTP, both in auditory inputs to the lateral amygdala (Abdou et al., 2018; Nabavi et al., 2014), and in hippocampal inputs to the nucleus accumbens (LeGates et al., 2018), while 1 Hz optogenetic stimulation induces LTD (Abdou et al., 2018; Kim and Cho, 2017; Sadegh Nabavi et al., 2014). Despite the evidence, it is possible that such stimulation protocols have different effects on the inputs to the BLA. In addition, the fact that AAV2-retro infects both local neurons and their inputs creates a scenario in which ChR2 is expressed both in the presynaptic and the postsynaptic neuron, which is different from previous reports, in which ChR2 was confined to the input neurons. The simultaneous expression in the pre- and post-synaptic side would suggest that any stimulation protocol would lead to potentiation, due to spike-timing dependent plasticity (Caporale and Dan, 2008; Dan and Poo, 2004). However, we observed that 1 Hz light stimulation reversed a contextual aversive memory generated by 100 Hz optogenetic stimulation (Figure 21). It has been shown that low frequency stimulation protocols (< 1 Hz) fail to induce LTP, even with the most favorable timing conditions (Caporale

and Dan, 2008; Sjöström et al., 2001). Besides, our setup induces simultaneous stimulation of both pre-and postsynaptic neurons, with virtually no interval between them, which has been shown to induce LTD at low frequencies (< 40 Hz) and LTP at high frequencies (> 40 Hz) in cortical neurons (Sjöström et al., 2001).

Our results showing sufficiency of synaptic potentiation of US-responsive BLA neurons could imply that potentiation of a high enough number of random BLA neurons would be enough to induce apparent aversive responses to previously neutral stimuli. Indeed, US exposure induces expression of CK2-DAA in a higher proportion of neurons than CS-only exposure (Figure 27). However, 100 Hz optogenetic stimulation of US-tagged BLA neurons and their inputs was insufficient to induce higher than baseline levels of freezing to tone or context (Figure 17), suggesting that the mere potentiation of a given proportion of BLA neurons is not enough to reproduce our results. Moreover, the BLA is an emotional center known to process both positive and negative valences (Namburi et al., 2016), therefore the potentiation of random BLA inputs might not be expected to result in a biased valence association. Distinct neurons in the BLA are known to project to different brain regions, which are associated with either positive, negative or neutral valences (Beyeler et al., 2016; Namburi et al., 2016, 2015). Indeed, we found that neurons tagged in response to the US-shock tend to project more heavily to the central amygdala, a well characterized component of the fear output circuit, than neurons tagged during CS exposure, which by comparison, displayed more projections to the nucleus accumbens, a known reward center (Figure 20).

3.2. Conclusions and future directions

The work presented here describes the identification of an engram for contextual fear conditioning, in the form of synaptic potentiation in learning-activated BLA neurons. First, we demonstrated that this form of plasticity occurs after context conditioning, but not after CS exposure or unpaired CS/US training (On Hab.), suggesting it is specific to the CS-US association (Figure 2). Next, we showed that reversing the synaptic potentiation in learning-activated BLA neurons impaired the memory, indicating that this form of plasticity is required for proper memory recall (Figure 8). Lastly, we demonstrated with two distinct strategies that artificial induction of synaptic potentiation between CS inputs and US neurons in BLA is sufficient to generate a *de novo* contextual fear memory (Figures 17 and 25). Therefore, we have provided substantial evidence to fulfill the three criteria for identifying an engram, as defined in Chapter 1 of this thesis (detectability, reversal and mimicry). I would argue that this work presents a complete set of evidence fulfilling the proposed criteria, thus, demonstrating that an observable learning-induced change is both necessary and sufficient to promote memory recall.

Previous studies show that context conditioning also induces plasticity in c-fos ensembles in different hippocampal subregions (Choi et al., 2018; Ryan et al., 2015), while lesions and pharmacological studies suggest it might occur in some neocortical regions as well (Corcoran et al., 2011; Frankland et al., 2004; Lesburguères et al., 2011). It would be interesting to use the techniques described here to manipulate plasticity in these regions to determine their role for context conditioning or other context-based tasks. Considering our results, I would expect that plasticity in these regions might modulate the response to context conditioning,

without being essential for the CS-US association. On the other hand, more complex context-based tasks might rely more on the associative capacity of these cortical regions.

As our results point to a specific subset of neurons in the BLA that is critical to forming a fear memory association, it would be interesting to determine the molecular identity of these neurons and the alterations induced by learning over time. Are the molecular changes limited to synaptic proteins? How is memory maintained over time? Do proteins with prion-like properties, or epigenetic mechanisms play a role in memory maintenance (Levenson and Sweatt, 2005; Si and Kandel, 2016)? The combination of techniques described here with single-cell sequencing, proteomics, RNAscope (Lake et al., 2016), among others, might provide answers to these questions.

Our results show that the c-fos+ ensemble activated during US exposure in the BLA undergo the critical plasticity that supports context conditioning. It is possible, however, that only a subset of this ensemble plays a critical role. Recently developed tools allow the genetic tagging of cells defined by the intersection of two or more features, such as IEG expression or connectivity (Fenno et al., 2014). Using these tools, it would be possible to refine the manipulation to a more specific BLA ensemble, such as neurons active by the CS+US but not by either the CS or US alone, or neurons activated during two different recall trials, in order to determine which neurons indeed are crucial to memory recall. Are the 15-25% of neurons active in any two sessions exploring the same environment [in the case of the hippocampus (Ziv et al., 2013)] the ones that are supporting memory recall?

Perhaps the major innovation provided by this thesis was to provide an approach to test the contribution of synaptic potentiation for memory storage with circuit specificity. This

approach could be readily adapted to dissect molecular and plasticity mechanisms behind other learned associations. We hope that our work will contribute to the investigation of memory beyond the current focus on the neuronal circuits involved, to include manipulation of learning-induced plasticity. This will advance the understanding of the mechanisms behind memory formation and storage. Dissecting the engram may also reveals targets of clinical relevance for potential treatments of cognitive disorders, such as posttraumatic stress disorder.

As Penfield stated, “A change of method or technique accounts for many things in human history” (Penfield, 1977). I am certain that the advent of new tools that allow genetic tagging and manipulation of specific neurons is the main reason why we were able to identify an engram for context conditioning, something that was so elusive for Karl Lashley and others decades ago. True scientific advancement comes from the combination of conceptual frameworks that change our perspective on natural phenomena with the development of tools that allow us to explore previously unsolvable questions. I believe that neuroscience will continue to rely on new tools as the main fuel source to unravel the mysteries of the brain.

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Chapter 4: Methods

All outstanding work, in art as well as in science,
results from immense zeal applied to a great idea.

Santiago Ramón y Cajal,
Advice for a Young Investigator, 1897

Animals

All animal procedures were conducted in accordance with institutional guidelines and protocols approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute and the University of California San Diego. All mice were bred in house on a C57BL/6NTac background (purchased from Taconic Biosciences), genotyped, housed 2-5 per cage, and maintained on a 12 h-light/ 12h-dark cycle with water and food *ad libitum.* Mice were 12-20 weeks old, in balanced male and female groups, although no significant difference in behavior were found among sexes.

The c-fos-shEGFP transgenic mouse line (Figures 1 and 3), which expresses a short-half life EGFP (2h) under the control of the c-fos promoter, was obtained as described previously (Reijmers et al., 2007), and it is available from The Jackson Laboratory, stock # 018306.

The double transgenic Arc-tTA x TetO-H2BGFP mouse line (Figures 4 and 5) was obtained by crossing the Arc-tTA line, in which the tetracycline transactivator (tTA) was knocked in the Arc gene, with the TetO-H2BGFP lina, which expresses a GFP fusioned to the human histone 1, H2bj, protein under the control of the tetO promoter (The Jackson Laboratory, stock # 005104). The histone-bound GFP is very stable and lasts for weeks (Tayler et al., 2013)

This mouse line was based in the TetTag system (Reijmers and Mayford, 2009), in which the activity-dependent gene (in this case, Arc) controls the expression of tTA, which binds to the tetO promoter, driving the expression of H2B-GFP. Another mouse line that was based on the TetTag system was the double transgenic c-fos-tTA x tetO-ChEF mice (Figure 19) generated by crossing single transgenic c-fos-tTA (Reijmers et al., 2007) and tetO-ChEF-tdTomato mice (Cowansage et al., 2014). For behavior experiments, single transgenic littermates were used for control. The Channelrhodopsin variant ChEF, which is more reliable at higher frequencies (Lin et al., 2009), is expressed in c-fos+ neurons during a time window control by the absence of the drug Dox, which sequesters tTA and inhibits it to bind to TetO and drive ChEF-TdTomato expression. In order to restrict activity-dependent labeling to targeted behavior episodes, mice were born and raised on food containing doxycycline (On Dox, at 40 mg/kg). Fours days prior to experimental manipulation (Three days for Arc-tTA x tetO-H2BGFP mice), mice were switched to food without Dox (Off-Dox) to allow the clearance of the drug before tagging. Mice were put back On Dox food 6h after behavior, first at a concentration of 200 mg/kg to quickly stop H2B-GFP expression, and after 12h, back to 40mg/kg for maintenance for the remainder of the experiment.

The c-fos-DD-Cre mouse (FDC), was generated by a knock-in of the DD-Cre fusion protein in the c-fos gene (Dillingham et al., 2019). This mouse line expresses a Cre-recombinase fused to a destabilizing domain (DD), which leads to protein degradation (Sando III et al., 2013). The drug trimethoprim lactate (TMP) stabilizes the complex, providing a window of functional Cre expression. TMP peaks in the brain in 10min and it is rapidly cleared, limiting the window of Cre expression to a few hours (Dillingham et al., 2019; Sando III et al., 2013).

Trimethoprim preparation and injection

Trimethoprim lactate (TMP) was purchased from Chem-Impex Int´l (# 03552), Inc in a lyophilized form and dissolved in H_2O to a final concentration of 20 mg/ml. Immediately before injections, this solution was mixed with 2X phosphate-buffered saline (PBS) to a final concentration of 10 mg/ml. Each animal was injected with 150 μg/g for TMP to reach peak concentration in the brain (Dillingham et al., 2019; Sando III et al., 2013). Intraperitoneal injections (IP) were given 20-30min after behavior, to minimize interference in conditioning and maximize labeling (Dillingham et al., 2019).

Viral constructs

All rAAVs were made in house with the DJ serotype (Grimm et al., 2008), except for ChR-retro (pAAV-EF1α-DIO-hChR2(H134R)-EYFP), which was acquired from Addgene (plasmid # 20298) with the rAAV2-retro serotype (Tervo et al., 2016) to target BLA and its projection neurons (Figure 13).

The rAAV-DJ production was carried out following protocol adapted from previous work (McClure et al., 2011). With the exception of Syn-GFP (see below), all rAAVs made in house contain the CaMKIIα minimal promoter (CK2min), favoring viral expression in excitatory neurons.

The recombinant AAV vectors used for viral production were:

- DIO-WT-CK2: pAAV-CK2min-DIO-CaMKIIα- -2A-H2BGFP
- DIO-CK2-D: pAAV- CK2min-DIO-CaMKIIα-T286D-2A-H2BGFP

- DIO-CK2-DAA: pAAV- CK2min-DIO-CaMKIIα-T286D/T305A/T306A-2AH2BGFP
- Timer: pAAV-CK2min-H2B-Timer
- DIO-Timer: pAAV-CK2min-DIO-H2B-Timer
- DIO-Syn-Venus: pAAV-Ef1α-DIO-Synaptophysin-Venus (provided by Anton Maximov's lab)

AAVs made in house were tittered through qPCR. The AAV vectors were injected in the BLA with the following titers*#:

- DIO-ChR-retro: 1.0 x 10^{13} units/ml genome copies (GC)/ml
- DIO-WT-CK2, DIO-CK2-D and DIO-CK2-DAA: 4 x 10^{12} GC/ml
- Timer: 8 x 10^{12} GC/ml
- DIO-Timer: 4.5 x 10^{13} GC/ml
- DIO-Syn-Venus: 1.1 x 10^{13} GC/ml

*: A minute volume (~1:100) of the dye Fast Green FCF (Tokyo Chemical Industry, # F0099) diluted at 1mg/ml in H_2O was mixed to the virus aliquot before injection to aid in visualization during surgery.

#: Whenever a combination of viruses was used (e.g. Figure 14), their concentrations were adjusted to match (1:1), and further dilutions were avoided.

Surgical procedures

For all surgeries, mice were first anesthetized with 5% isoflurane/oxygen mix in an induction chamber (20x10x8 cm) until proper sedation was achieved. Animals were transferred

to stereotaxic apparatus (Kopf Instruments, Model # 1900), and anesthesia was maintained with 1.5-2% isoflurane/oxygen mix. Hair was removed from the head and the skin disinfected with 70% ethanol followed by the application of 9% Povidone-iodine solution (Betadine) and again 70% ethanol to remove excess of Betadine. A small incision was then made on the scalp and the skin carefully retracted to allow direct access to the skull. Body temperature was maintained using a deltaphase isothermal pad (Brain Tree Scientific, Inc.). Veterinarian petrolatum ointment (Puralube) was used to prevent eyes from drying during surgery. After the surgery, a subcutaneous injection of 2.5 µg/g flunixin (Sigma-Aldrich, # 33586) was given to reduce post-surgical pain and inflammation. After surgery, mice were allowed to recover for 7d before any further experimental procedure.

AAV injection: Small holes were drilled to the skull, and the appropriate AAV was delivered bilaterally (unless otherwise specified) to BLA using a thin glass needle (Sutter Instrument, #BF100-50-10) of 30-40 µm tip diameter at the following Bregma coordinates: -1.45 mm anteroposterior (AP), ±3.32 mm mediolateral (ML), -4.9 mm dorsoventral (DV). The definition of Bregma was the (often imaginary) intersection between the sagittal suture and the curved formed by the coronal suture, similar to previously described in rats (Blasiak et al., 2010). Injection volume was 300nl per hemisphere, enough to achieve 70-100% coverage of BLA, except for ChR-retro and Syn-Venus, in which 200nl was used to avoid any off-target labeling, achieving 50-70% coverage of BLA. The injection speed was kept at 100nl/min using a infusion pump (Pump 11 Elite Series, Harvard Apparatus) connected to the glass needle through a 2.0 µl syringe (Hamilton Company, #88500) and hard plastic tubings filled with Mineral Oil (Fisher Scientific, BP26291). The glass needle was left in place 3min prior to injection and 5min following infusion to minimize backflow. At the end of the surgery, if no

optic fiber placement was required, the skin was sutured back in place with sterile absorbable surgical suture (Ethicon, # J844G).

Optic fiber placement: Prior to optic fiber placement, two screws (Plastics One, # 00-96X1-16) were added to the skull to provide extra support and stability at -3.5mm AP, -1.5mm ML and -0.85mm AP, -1.3mm ML. 200µm-core optic fibers (NA 0.37) connected to ceramic ferrules (Newdoon, FOC-C-200-1.25-0.37-5.0), were placed at -1.45mm AP, ±3.32 mm ML, -4.6 mm DV (300µm above the center of AAV injection in the case of ChR-retro). Dental cement (Stoelting, # 51458) was then added to secure the optic fibers in place, forming a head cap.

Slice electrophysiology

Following training at specified time points, mice were anesthetized with isoflurane drops in a glass jar followed by decapitation. Brains were quickly removed and placed in ice-cold N-methyl-D-glucamine (NMDG) based recovery solution containing (in mM): NMDG 93, HCl 93, KCl 2.5, NaH_2PO4 1.2, $NaHCO_3$ 30, HEPES 20, Glucose 25, sodium ascorbate 5, thiourea 2, sodium pyruvate 3, $MgSO_4$ 10, $CaCl_2$ 0.5) (Ting et al., 2014). Coronal slices (300 µm) were cut in a vibratome (Compresstome VF300) and incubated in the same NMDG solution for 10-15 min at 32°C before transferred to a HEPES aCSF solution containing (in mM): NaCl 92, KCl 2.5, NaH_2PO4 1.2, $NaHCO_3$ 30, HEPES 20, Glucose 25, sodium ascorbate 5, thiourea 2, sodium pyruvate 3, $MgSO_4$ 10, $CaCl_2$ 0.5 at room temperature for an additional hour before recording. Recording was conducted in an open bath chamber continuously perfused with warm (30–32°C) aCSF containing (in mM): NaCl 124, KCl 2.5, NaH_2PO4 1.2, $NaHCO_3$ 24, HEPES 5, Glucose 12.5, $MgSO_4$ 2, $CaCl_2$ 2, at the rate of 2–3 mL/min. Olympus

BX51WI upright microscope (Scientifica) was used for viewing the slices in differential interference contrast with 60× magnification. Identification of GFP+ cells was conducted with a 470nm LED (CooLED) and a CCD camera (ORCA-Flash4.0LT, Hamamatsu).

Recordings of BLA pyramidal cells were performed with glass micropipettes (resistance 3.5-5 MΩ) pulled by a PC-10 puller (Narishige) and filled with intrapipette solution containing (in mM): 120 Caesium methansulphonate, 20 HEPEs, 0.4 EGTA, 2.8 NaCl, 5 tetraethylammonium chloride, 2.5 MgATP, 0.25 NaGTP (pH 7.3, 285 mOsm) (Namburi et al., 2015). 2 mM Alexa Fluor 494 was added to the intrapipette solution for a subset of cells. For measuring AMPAR/NMDAR ratio, brain slices were perfused with a γ-aminobutyric receptor A ($GABA_A$) antagonist picrotoxin (100 μM, Tocris). A concentric bipolar stimulation electrode (FHC) was placed in the internal capsule fibers projecting to the basolateral amygdala. Stimulation intensity ranged from 50-200 μA. Cells were voltage clamped at +40 mV and evoked EPSCs were recorded every 10 sec. Once a stable baseline of EPSCs (compound AMPAR+NMDAR current) was established, an NMDAR antagonist AP5 (50 μM, Tocris) was applied for 5 min and AMPAR EPSCs were recorded in the presence of AP5. NMDAR current was obtained by subtracting the AMPAR EPSC from the compound EPSC. Average of 10-15 EPSC traces were used. Paired pulsed ratio (PPR) of evoked EPSC amplitudes was measured following two stimulations separated by 50 msec.

For measuring intrinsic properties and excitability, intrapipette solution containing (in mM): 123 K-gluconate; 2 MgCl2; 8 KCl; 0.2 EGTA; 10 HEPES; 4 Na2-ATP; 0.3 Na-GTP was used. Resting membrane potential was measured at current clamp when whole-cell patch was established. Depolarization currents of increasing amplitude (10 pA steps, 250 msec) were injected into the cell through the patch-pipette in current clamp mode. The action potential (AP)

evoked by the smallest current injection was used for measuring threshold, amplitude, and half width. Input resistance was estimated in the linear portion (-50 pA to 50 pA) of the current-voltage plots. Repetitive spiking was measured using a series of 250 msec current steps (-50 pA to 250 pA, 50 pA/step). The input-output relationship between the current size and action potential numbers was constructed and the latency to the 1st spike at 250 pA current injection was measured. Post-burst afterhyperpolarization (AHP) was measured at -5 mV below the AP threshold and AHP was evoked using a 100 msec current step that elicits 4 APs (McKinney et al., 2009). For mEPSCs, cells were held at –70 mV in voltage-clamp mode and recorded in the presence of tetrodotoxin (0.5 μM) and picrotoxin (100 μM; Tocris) for 5 min. Cells which access resistance were >20 MΩ or that varied more than 30% during the recording were excluded from analysis. Each group contained 5-9 cells from 4-6 mice.

Multiclamp 700B amplifier and pClamp10 software were used for data acquisition (filtered with 2 kHz low pass filter) and digitization (10 kHz sampling frequency). Electrophysiological data were analyzed by Clampfit, Mini Analysis and Igor.

Handling

All behavioral experiments were conducted during the facility light cycle (6:00 am to 6.00 pm). All mice were individually habituated to the investigator by handling for one minute on each of five consecutive days. Handling took place in the mouse colony. Immediately prior to each handling session mice were transported by wheeled cart to and from the vicinity of the experimental context rooms to habituate them to the journey.

Fear Conditioning

Tone and Context conditioning were carried out in MedAssociates boxes (box A, see below). Unless otherwise specified, each session lasted for 6 min, starting with 3 min acclimation to the context, followed by three 30-second-long tones (5 kHz, 70 dB) starting at 180 sec, 240 sec and 310 sec, that were co-terminated with 1 sec 0.6 mA foot shocks.

For context conditioning only, the same 6-min protocol was used, but no auditory tones were played. 1 sec 0.6 mA foot shocks were delivered at 209 sec, 269 sec and 339 sec. For CK-DAA US tagging (Figure 24), 1.5 mA foot shocks were used to promote labeling of US-tagged neurons.

For context conditioning with unpaired tones (Figure 1), the same auditory tones (30 sec, 5 kHz, 70 dB) and foot shocks (1 sec, 0.6 mA) were given, but following a different schedule: tones started at 100 sec, 150 sec, and 210 sec and foot shocks were given at 270 sec, 300 sec and 340 sec.

For overnight habituation (ON), animals were introduced to the box 16 h before shock exposure, 2 h before the end of the light cycle. Food and water (hydrogel) were provided, and the 12 h-light/ 12h-dark cycle was followed. On the next day, 2h after the beginning of the light cycle, the correspondent behavior was carried out. The long habituation to the box inhibits conditioning with foot shock exposure (Impey et al., 1998).

Box exposure lasted for 6min, with no shock or tone, unless otherwise specified. Tone exposure in Box B was played through in iPad calibrated with a sound level meter (MedAssociates,

ANL-929A-PC) using the Tone Generator Pro app. Auditory tones were delivered at the same time as in tone conditioning: three 30-second-long tones (5 kHz, 70 dB) starting at 180 sec, 240 sec and 310 sec.

Context recalls were 180 sec long, while Tone Recalls were 220 sec long, with a single tone (30 sec, 5 kHz, 70 dB) starting at 180 sec.

Freezing behavior, defined as the absence of all movement except that required for respiration, was first manually counted for ~20% of animals in every experiment in order to adjust the threshold used in the automated system to give the final counts for all animals. The MedAssociates system was used for box A and tone recall, and boxes B and C were recorded with a camera (Logitech C270 HD Webcam) mounted above them and subsequently analyzed using an automated video tracking software (ANY-maze, Stoelting Co.).

Context extinction: For context extinction (Figure 5), animals were exposed to the conditioning box (box A) for 30 min/day during five consecutive days. The reported freezing during extinction trials consists of the first 3 min of each session. Retraining was carried out using the same protocol as for novel context conditioning.

Contextual environments: Box A, B and C were located in different rooms, with different illumination patterns to help with discrimination among contexts.

Box A consisted of a rectangular box (25.5 x 29 x 25.5 cm) with metal rod grid floor, two metal sidewalls, one black and white checkerboard back wall, and transparent Plexiglas front wall and ceiling. A piece of paper with peppermint odor was kept at the vicinity of the box to provide a distinct odor cue. A direct fluorescent-white light illuminated the box and the room lights were on. Box A exposures were carried out inside the MedAssociates chamber.

The box was cleaned with 70% ethanol between sessions. All conditioning and foot shock exposures were carried out in Box A.

Box B consisted of a white acrylic square box (25.5 x 25.5 x 25.5 cm) open on top. A piece of paper with lavender odor was kept at the vicinity of the box to provide a distinct odor cue. The box was cleaned with 70% ethanol between sessions. An indirect fluorescent-white light illuminated the box and the room lights were on.

Box C consisted of a gray opaque plastic box (40 x 20 x 25 cm) open on top. A piece of paper with orange odor was kept at the vicinity of the box to provide a distinct odor cue. The box was cleaned with 70% ethanol between sessions. An indirect fluorescent-white light illuminated the box and the room was dimly illuminated.

Tone recall was carried out in the Med Associates chamber. To make it distinct from box A, the whole chamber (25.5 x 29 x 25.5 cm) was covered in white plastic walls and floor, except for the ceiling and the transparent Plexigas front wall. Mice were put inside a smaller transparent plastic box (19 x 17.8 x 17.8 cm) with fresh bedding on the floor, and this box was inserted into the bigger chamber. A piece of paper with cinnamon odor was kept at the vicinity of the box to provide a distinct odor cue. The box was cleaned with 70% isopropanol between sessions. A direct fluorescent-white light illuminated the box. The room was dimly illuminated and a black curtain was used to make it seem smaller.

Anxiety tests

The anxiety tests were carried out in consecutive days, starting with the elevated plus-maze, the open field test, and finishing with the marble burying test.

Elevated plus-maze (EPM): Mice were tested in an EPM that was elevated 75 cm from the floor by 6 legs, following standard protocol (Walf and Frye, 2007). Each arm measured 30 x 5 cm and closed arms had walls of 30 cm. Animals were always placed at the center of the maze, facing the open arm opposite to where the experimenter was, and let to explore it for 5min. The maze was cleaned with 70% ethanol before each session. Behavior was recorded using a camera mounted above the arena (Logitech C270 HD Webcam). Animal movements and position were subsequently analyzed using an automated video tracking software (ANY-maze, Stoelting Co.) to determine freezing (see above), the travelled distance, and time on open area (center + open arms).

Open field test: Mice were place in the center of an open arena (50cm x 50 cm) and let to explore it for 5min. A piece of paper with vanilla odor was kept at the vicinity of the box to provide a distinct odor cue. The arena was cleaned with 70% isopropanol before each session. Behavior was recorded using a camera mounted above the arena (Logitech C270 HD Webcam). Animal movements and position were subsequently analyzed using an automated video tracking software (ANY-maze, Stoelting Co.) to determine freezing (see above), the travelled distance, and time spent at the center or edges of the open arena (center defined as square of 30 x 30 cm).

Marble burying test: The test was conducted as described elsewhere (Deacon, 2006). Briefly, mouse cages were filled approximately 5 cm deep with wood chip bedding, and 10

multi-colored opaque glass marbles were evenly arranged on top of it. A single mouse was carefully put in each cage, not on top of any marble and the cage was closed. Each test session lasted for 30 min and buried marbles (threshold was 2/3 buried) were manually counted. The duration of the test was determined by preliminary experiments with wild-type mice that have been fear conditioned; 30 min was the average time required to bury half of the marbles.

Optogenetic stimulation

All optogenetic stimulation sessions were carried out using a 473 nm diode-pumped solid-state (DPSS) laser (Laser Century). Blue light was delivered bilaterally in BLA through 200µm-core optic fibers (NA 0.37) connected to ceramic ferrules (Newdoon, FOC-C-200-1.25-0.37-5.0). Laser intensity at the tip of the fiber was set to 10 mW.

100 Hz optogenetic stimulation: Our protocol was based on what has been previously established to induce LTP in auditory inputs to LA (Abdou et al., 2018; Nabavi et al., 2014). Mice were attached to the optic fiber patch cords and allowed to individually explore an open mouse cage with fresh bedding for 5 min. Optogenetic stimulation consisted of 5 trials of light with 3 min inter-trial interval. Each trial consisted of 2 ms, 100 Hz pulsed light with a total duration of 1 sec (20% duty cycle). 3 min after the end of light stimulation, the patch cord was detached, and the mouse was returned to its home cage.

1 Hz optogenetic stimulation: Our protocol was based on what has been previously established to induce LTD in auditory inputs to LA (Abdou et al., 2018; Kim and Cho, 2017; S Nabavi et al., 2014). Mice were attached to the optic fiber patch cords and allowed to individually explore an open mouse cage with fresh bedding for 5 min. Optogenetic stimulation

consisted of 900 pulses of light, each 2 ms, at 1 Hz (0.2% duty cycle). 3 min after the end of light stimulation, the patch cord was detached and the mouse was returned to its home cage.

20 Hz optogenetic stimulation: Our protocol was based on what has been previously reported for driving freezing behavior (Liu et al., 2012), using parameters tested in the BLA (Gore et al., 2015). Mice were attached to optic fiber patch cords and placed at the center of a novel circular white plastic box with 25 cm of diameter and 20 cm high walls. A piece of paper with ginger odor was kept at the vicinity of the box to provide a distinct odor cue. The protocol started with 3 min of baseline, followed by 3 min of Light ON, 3 min Light OFF and 3 min Light ON, for a total of 12 min. The Light ON periods consisted of Light pulses at 20 Hz, with 10 ms Pulse width (20% duty cycle).

c-Fos immnohistochemistry

90 min following the last behavioral session, mice were overdosed with isoflurane and transcardially perfused with 1x PBS followed by 4% paraformaldehyde (PFA) in 1x PBS. Brains were removed and kept in 4% PFA 1x PBS at 4°C overnight. On the following day, brains were washed in 1x PBS and sliced at 75μm using a Leica VT100S vibratome. Immunohistochemistry was performed in free floating slices, and all the incubations were performed in horizontal shaker at gentle speed. Brain slices were first incubated for 3 h in blocking solution (10% normal goat serum [Jackson Immuno Research, 005-000-121], 0.2% Triton X-100, 0.05% Sodium azide) at room temperature. Following this step, slices were incubated for 12-16 h in blocking solution containing 1:700 rabbit anti-c-Fos antibody (Cell Signaling Technology, #2250S) at 4°C. After three 10 min washes in 0.2% Triton 1x PBS

solution, slices were incubated for 3-4 h in blocking solution containing 1:750 goat anti-rabbit antibody conjugated with Alexa dye 647 (Invitrogen, #A-21244) at room temperature. After one wash in 0.2% Triton 1x PBS solution, slices were incubated for 30 min with 200μM DAPI (Invitrogen) diluted in 0.2% Triton 1x PBS solution for counterstaining. Slices were then washed three times in 1x PBS and mounted in microscope slides (Fisherbrand Superfrost Plus, Fisher Scientific) with antifade mounting medium (SlowFade Gold, Invitrogen). Imaging was carried out within a week.

Confocal microscopy and cell counting

All brains, regardless of undergoing immunohistochemistry or not, were fixed in 4% paraformaldehyde overnight, washed in 1x PBS and cut using a Leica VT100S vibratome (50-75 μm slices). Slices were counterstained with DAPI (Invitrogen) and mounted in microscope slides (Fisherbrand Superfrost Plus, Fisher Scientific) with antifade mounting medium (SlowFade Gold, Invitrogen). Initially, slides were counterstained with Hoechst 33258 (Invitrogen), which made it easier to distinguish the BLA from surrounding amygdalar nuclei but make it more difficult for automated cell counting due to staining of fibers.

Images were acquired using a Nikon A1 R+ confocal microscope with 4x (0.2 NA, overview images) and 20 X (0.75 NA, detailed images for cell counting) plan apochromat objective lens and lasers at 405 nm, 488 nm, 561 nm and 640 nm. All acquisition parameters were kept constant within each magnification for a given experiment. All cell counting experiments were conducted blind to experimental group, using 3-4 coronal slices per region of interest for each mouse (slices with the most viral labeling were chosen). Cell counts and

overlap quantification automatically were completed using an ImageJ macro which identifies only cells that overlap with DAPI (available upon request). The reactivation of neurons tagged with CaMKIIα AAVs (GFP+), which has been shown to correlate with memory retrieval (Reijmers et al., 2007; Tayler et al., 2013), was calculated as the number of cells that are both c-fos+ (IHC) and GFP+, divided by the total number of GFP+ cells (Figures 9 and 27).

For synaptophysin-Venus (Syn-Venus) AAV experiments, regions of interest were drawn around the CeA, NaC or PFC in ImageJ, and the total area was measured. Next, all images were thresholded to include only the brightest signal (by eye), and the thresholded signal area was measured. The signal area (Syn-Venus) / total area was averaged for each mouse before averaging each group. Such measurement was found to correlate well with the number of labeled axonal boutons counted in images with higher resolution (Dillingham et al., 2019).

Statistics

Behavioral data analysis and statistics were conducted using Prism (Graphpad). Electrophysiological data analysis and statistics were conducted using Clampfit and Igor. Data from two groups were compared using two-tailed unpaired Student's t-tests. Multiple group comparisons were assessed using one-way or two-way repeated-measures (RM) ANOVA with post hoc tests as described in the appropriate figure legend. Data are presented as means ± the standard error of the mean (SEM).

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