Removal and reinstatement of a memory with LTD and LTP

A thesis submitted in partial satisfaction of the requirements for the degree master of science in Biology

by

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The thesis of Rocky Garrett Fox is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

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by

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Master of Science in Biology

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It has been long believed that long-term potentiation (LTP) and long-term depression (LTD) are the primary mechanisms for encoding memory. However, causal evidence of this has thus far been elusive. Here we demonstrate a causal relationship between these mechanisms of plasticity and associative fear memory in rats. Using optogenetic targeting of auditory inputs to the lateral amygdala, an associative memory can be formed through light stimulation paired
with foot-shock. Following this conditioning, fear memory can be activated through light stimulation as demonstrated by freezing and avoidance of a strongly rewarding task. LTD induction through low frequency stimulus leads to removal of this memory, demonstrated by continued reward task activity during light stimulation. Subsequent LTP induction through high frequency stimulation leads to restoration of the memory, as shown by restored freezing and reward task avoidance during light stimulation. Using this simple series of experiments we have demonstrated the removal and reinstatement of a memory using LTD and LTP, thus demonstrating a causal relationship between these synaptic processes and memory.
I:

Introduction
How memories are stored has been one of the premier questions in neuroscience for the past 60 years. Since Hebb’s synaptic modification postulate in 1949, the idea that memories are formed through modification of synaptic weights has persisted and evolved. In the following decades neuroscientists identified, using electrophysiology, two processes for modifying synaptic strength in the hippocampus, long-term potentiation (LTP) and long-term depression (LTD). Both of these processes, which are primarily NMDA receptor dependent, have been major targets in the study of neuronal plasticity. Since the initial discovery of LTP in the rabbit hippocampus\(^1\), glutamatergic synapses able to undergo these types of plasticity have been found throughout the brain.

LTP can be induced by one of three methods, however only two will be discussed here (the third, theta burst is not addressed in this study). The first occurs when presynaptic axons receive brief, high frequency stimulation causing a subsequent large release of glutamate to the synaptic cleft, which leads to a post-synaptic depolarization and up regulation of the AMPA-type glutamate receptor. The second occurs associatively when a weak low frequency synaptic stimulation is paired with the depolarization of the post-synaptic cell also leading to the up regulation of AMPAr’s at the stimulated synapse. Both of these methods lead to LTP and an increase in synaptic transmission between these two neurons. It is this “strengthened” connectivity that is believed to be the foundation of memory encoding.
Conversely, LTD is induced using sustained low frequency stimulation of presynaptic inputs, which leads to a down regulation in post-synaptic AMPAr’s. This down regulation of receptors leads to a reduction in synaptic transmission. This reduction in level of connectivity could be involved either in the process of forgetting, disassociation or simply a modification to complement LTP in order to control signal-to-noise ratio and increase storage capacity.

Many strides have been made in the quest to connect these processes of bidirectional plasticity with memory. Potentiation occurring during memory formation has been demonstrated by in vivo recording in relevant brain regions following learning. The necessity of these changes in plasticity for memory formation have been shown indirectly through NMDA receptor blockade, as well as genetic manipulation of AMPA receptors, both of which perturb memory formation and learning. Additionally, a complete potentiation (or saturation) of synapses crucial to a specific type of memory has been shown to occlude memory formation.

While each of the experiments above when taken together provide compelling indirect evidence of the role of plasticity in memory, a direct causal link has thus far been difficult to demonstrate. The major difficulty in establishing this causal link has been that many of the studies addressing plasticity and memory have focused on hippocampal dependent memory. The overwhelming complexity of hippocampus circuitry and spatial memory, as well as the fact that hippocampal dependent memories are not ultimately stored in the
hippocampus, has made demonstrating a direct causal link extremely difficult\textsuperscript{2}.

To address this problem we looked to the amygdala, and a simple form of associative memory, auditory cued-fear conditioning\textsuperscript{8–13}. By targeting this circuitry using optogenetics we show that a depression of transmission (LTD) at synapses undergoing potentiation during to fear conditioning can be used to remove a memory, which can then be reinstated by re-potentiating (LTP) this same group of synapses, thereby demonstrating a direct causal link between LTP and memory formation.
II:

Results
In order to examine the role of synaptic plasticity in memory we chose to target auditory fear conditioning\textsuperscript{8–13}, a form of associative memory, whereby a conditioned stimulus (CS, a tone) is temporally paired with an aversive unconditioned stimulus (US, a foot shock) during the training phase. Following training, presentation of the CS alone produces the conditioned response, namely behavioral fear response in the form of freezing.

While freezing is an adequate measure of fear, sensitivity can be low and results inconsistent due to high background levels of freezing. In order to increase the sensitivity of the fear measurement we chose to present the CS during a rewarding task\textsuperscript{14}. Rats were trained to press a lever to receive a small amount of \%10 sucrose solution as a reward. This task kept the rats continually active pressing the lever during testing. In this paradigm, fear was recorded as a reduction in lever presses. We found that this assay correlated well with freezing (R\textsuperscript{2}=0.4, p<0.05; Fig. 7), displayed much greater sensitivity (Fig. 7 legend) and did not require observer-assessed freezing. Rats conditioned by temporally (but not non-temporally) pairing tone and shock displayed a large decrease in lever press activity during tone exposure in subsequent testing (Fig. 1a).

The lateral amygdala is the main site of associative plasticity for auditory cued fear conditioning\textsuperscript{8}. It receives two primary inputs for auditory CS, the first, from the medial geniculate nucleus (MGN) of the thalamus, and the second, from the auditory cortex (AC). By infecting these two areas with adeno-associated virus (AAV) expressing oCHIEF\textsuperscript{15} (a light activated channelrhodopsin-2 variant
with effective kinetics up to 50-100Hz), waiting 3 to 4 week for sufficient
eexpression of channels at axon terminals, and implanting a fiber optic cannula for
light delivery with its tip just above the lateral amygdala, the auditory CS input to
the lateral amygdala can be replaced by optogenetic stimulation. Controlling the
duration and frequency of neurotransmitter release using optical stimulation in
vivo, by specifically targeting inputs to the amygdala, allows us to test the effects
of synaptic plasticity on memory (i.e. conditioned response) directly.

As a neutral conditioned stimulus we used a 10 Hz optical stimulation
(2ms 15-20 mW illumination) for 2 minutes, which produced no effect on rate of
lever press (Fig. 9). Animals then underwent unpaired conditioning, whereby a
10 Hz light stimulus was given temporally unpaired with foot-shock (see
methods). Following this conditioning, animals displayed no reduction in lever
pressing in subsequent testing (Fig. 1b). Animals were then given multiple paired
conditioning periods, consisting of 1 second, 10Hz light stimuli co-terminating in a
foot-shock. During testing 1 and 2 days later, these animals showed significant
reduction in lever pressing during optically driven input (ODI) stimulation (Fig.
1b). Because cannula location and adequate viral expression could not be
verified a priori, animals were screened following paired conditioning. Animals
that did not display a reduction in lever presses during ODI stimulation were
examined histologically and showed either misplaced cannula or insufficient viral
expression in auditory inputs to the lateral amygdala (Fig. 10). The
administration of NMDA receptor antagonist (MK-801) during paired conditioning
prevented ODI stimulus induced conditioned response during testing (Fig. 11). These results indicate that a form of associative memory is produced through the pairing of ODI stimulation with shock.

After demonstrating that an associative memory can be formed through the pairing of ODI stimulation with shock, we wanted to see if a reduction in synaptic transmission from auditory inputs to the amygdala, induced by an LTD protocol, was sufficient to prevent a future ODI induced conditioned response. Animals that displayed a conditioned response to stimulation of ODI underwent an LTD protocol, whereby a 1 Hz light stimulus was delivered for 15 minutes (900 pulses). In subsequent testing these animals displayed no conditioned response to 10 Hz light stimulation (Fig. 2b). Following this removal of ODI activated memory (conditioned response) through the use of an LTD protocol, we wished to test if this memory could be reinstated through the use an LTP protocol. This same group of animals underwent an LTP protocol, consisting of 5 trains of 100 Hz light stimulation each separated by 3 minutes\(^{16}\), shortly after post-LTD testing. 24 hours later the animals displayed the same conditioned response seen following paired conditioning (Fig. 2c), suggesting a reinstatement of memory. In order to verify that these LTD and LTP protocols (which are analogous to those in vitro) could have the same effect in vivo as they do in vitro, and undergo multiple rounds of bidirectional plasticity, we induced a second round of LTD and LTP. In each case, subsequent 10 Hz testing 24 hours after each protocol showed a removal and then reinstatement of conditioned response to ODI stimulation for
LTD and LTP, respectively (Fig. 2d,e). The effects of these protocols were rapid and long-lasting (Fig. 12).

To further understand how this conditioned response is induced by the ODI, we ran experiments to test the effects of plasticity protocols on naïve animals. To parallel the protocol given in Fig. 2, we began by inducing LTD and testing the effect of ODI stimulation, followed by LTP induction, and again testing the effects of ODI stimulation. In each case, the plasticity protocol had no effect on lever pressing (Fig. 3a,b,f). These animals then underwent paired conditioning, after which they displayed a conditioned response to ODI stimulation. This ODI induced conditioned response could then be removed and reinstated by LTD and LTP protocols (Fig. 3c,d,e), as before. Next we induced just LTP on another group of naïve animals, and they also displayed no effect of light during subsequent 10 Hz testing (Fig 4a). Again, following paired conditioning, these animals displayed the same conditioned response to 10 Hz light that could then be removed and reinstated using LTD and LTP protocols (Fig. 4b-e). These experiments show that an LTP protocol can only reinstate a conditioned response that has been previously formed through the pairing of ODI with shock, and subsequently removed by LTD induction. An LTP protocol delivered to the ODI on its own is not sufficient to drive a conditioned response.

To further investigate the effects of bidirectional plasticity modifications on memory we tested the effects of LTD and LTP protocols on auditory cued-fear conditioning. We first tested to see if LTD applied to the ODI could inactivate
tone-induced fear conditioning. In two groups of naïve animals we infected unilaterally the medial geniculate nucleus and auditory cortex with AAV-oChIEF, and pharmacologically ablated the contralateral amygdala using NMDA (See methods). The first group was pair conditioned to associate a tone with shock (see methods), and the next day displayed the expected conditioned response to tone (Fig. 6a,d). The second group received the same pair conditioning protocol immediately followed by an LTD protocol applied to the ODI. The following day the second group of animals displayed a significant reduction in conditioned response (Fig. 6c,d). These experiments are consistent with the view that memories are formed through the potentiation of synapses via LTP, which can then be removed by de-potentiation or LTD. Additionally, we tested to see if LTP applied to the ODI could reverse extinction of tone-induced fear. A group of animals that had received tone conditioning underwent an extinction protocol consisting of 6 sessions of 2 minutes of tone over three days in the absence of shock (see methods). These animals displayed no conditioned response to tone in subsequent testing (Fig. 6e). Following extinction, the animals underwent LTP and did not display a conditioned response 24 hrs. later (Fig. 6f). Due to a possible lack of sufficient overlap between the ODI’s to the lateral amygdala and those neuronal inputs active during auditory fear conditioning we wanted to test this paradigm on animals that had learned to associate shock with activation of ODI’s. Following ODI paired conditioning animals underwent 5 consecutive days of 2 minutes 10 Hz light testing until a reduction of lever presses was no longer
seen during ODI stimulation. The animals then underwent an LTP induction protocol, and 24 hours later no conditioned response was observed (Fig. 6h,i,j). These results are supportive of the idea that extinction is not merely a weakening of potentiated synapses in the lateral amygdala\textsuperscript{17}.

To confirm that light induced plasticity protocols of ODI’s were producing the expected post-synaptic effects, we conduct in vivo recording of ODI-evoked post-synaptic responses in the lateral amygdala of anesthetized animals expressing oCHIEF in auditory inputs (see methods). These ODI induced field responses in vivo resembled those light evoked synaptic responses recorded in vitro which could be eliminated using AMPA receptor antagonist, NBQX (Fig. 14). In vivo responses were evoked using a single light pulse every 30 seconds until a stable baseline was established. A 2-minute 10 Hz light stimulation protocol produced no lasting effect on post-synaptic light evoked responses (Fig. 5a). However, the same LTD protocol as that used during behavioral testing produced a lasting reduction in post-synaptic light-evoked responses (Fig. 5b). As expected, the LTP protocol used during behavioral testing produced a lasting potentiation of light-evoked post-synaptic responses (Fig. 5c). These protocols were able to reverse the effects of one another (Fig. 15), just as is observed in those analogous protocols in vitro. These results confirm that light induced plasticity protocols to the ODI produce the expected synaptic effects.

In order to verify that the pairing of ODI stimulation with foot-shock produced synaptic potentiation in the lateral amygdala we examined lateral
Amygdala acute slices taken from animals that had received no conditioning, unpaired conditioning and paired conditioning. We conducted whole-cell recording of lateral amygdala neurons and measured the postsynaptic response to optical stimulation of auditory inputs. By clamping membrane potential at -60 mV, measuring optically evoked (inward) current, and then clamping membrane potential at +40 mV, and measuring optically evoked (outward) current, the AMPAr (A) current (at -60 mV) and the AMPAr and NMDAr (N) current (at +40 mV) can be compared to the current at holding potential (+0 mV) and an A/N ratio can be calculated (see Methods). An increase in A/N ratio has been shown to be indicative of the occurrence of potentiation at synapses. We found that there was no difference between A/N ratio of optically driven synaptic inputs to amygdala neurons in animals that had received no conditioning (2.4±0.24; N=11) and animals that had received unpaired conditioning (2.1±0.18; N=10). However, animals that had received prior paired-conditioning displayed a marked increase in A/N ratio (4.4±0.63; N=8) when compared to both controls (p<0.001, Fig. 1c). These results demonstrate that the pairing of ODI stimulus with foot-shock causes potentiation of auditory synapses in the lateral amygdala.
Figure 1: Fear conditioning with tone or optogenetic input.

a, top, diagram of rat receiving tone and shock during conditioning. Rats were exposed to unpaired (N=5 naïve rats, middle) or temporally paired (N=5 naïve rats, bottom) tone and shock (see Methods), and tested one day later by delivery of a period of tone, where indicated (green shaded area). Time graphs plot number of lever presses, normalized to baseline period, to a previously learned cued lever-press task. Each point represents data collected over 1 minute. Error bars indicate SEM here and throughout. Bar graph indicates normalized number of lever presses during the first minute of tone.

b, Top, diagram of rat receiving intracranial optical stimulation of optogenetically driven inputs (ODI) and shock during conditioning. Rats (N=8) were exposed to unpaired (middle) and one day later temporally paired (bottom) stimulation of ODI and shock (see Methods). Time graphs plot number of lever presses, normalized to baseline period, to a previously learned cued lever-press task. Animals were tested one day after each conditioning by delivery of 10 Hz stimulation of ODI, where indicated (blue shaded area). Bar graph as in a for 10 Hz ODI.

c, top, experimental design; averaged synaptic responses obtained at -60 mV (blue), +40 mV (red) and 0 mV holding potential for cells from animals that received unpaired (top) or paired (bottom) conditioning. Bar graph plots average AMPA/NMDA for cells from the indicated groups.

d, Cellular models of synaptic modifications that can explain conditioned response following paired conditioning. Pairing of tone and shock inputs (left) or optogenetically driven inputs (ODI) and shock inputs (right) to lateral amygdala neurons leads to potentiation of tone input (left, red) or ODI input (right, red), such that subsequent activation of this input is sufficient to trigger downstream behavioral effects (fear reducing lever pressing).
Figure 2: LTD and LTP remove and reinstate memory.
A single group (N = 12) was tested for conditioned response (a) as measured by changes in lever presses normalized to baseline period, two days following paired conditioning of ODI and shock. Blue area indicates delivery of 10 Hz optical stimuli to ODI. After testing, animals were delivered an LTD protocol through the ODI. The same group was tested for conditioned responses (b) one day after LTD protocol. After testing, animals were delivered an LTP protocol through the ODI. The same group was tested for conditioned response (c) one day after LTP protocol. After testing, animals were delivered another LTD protocol through the ODI. The same group was tested for conditioned response (d) one day after second LTD protocol. e, Normalized lever presses one minute following 10 Hz ODI stimulation after different protocols (as indicated). f, Cellular models of synaptic modifications occurring in the lateral amygdala that can explain behavioral responses following LTD (top) or LTP (bottom) protocols delivered to ODI.
Figure 3: LTP produces conditioned response only after prior paired conditioning; protocol one.
A naïve group of animals (N=4) was tested for conditioned responses to 10 Hz ODI stimulation (shaded blue area) one day after LTD protocol (a), one day after subsequent LTP protocol (b), one day after subsequent paired ODI and shock conditioning (c), one day after subsequent LTD protocol (d) and one day after subsequent LTP protocol (e). f, Graph of normalized lever presses one minute during 10 Hz ODI stimulation one day following indicated protocols. Note that conditioned response is seen following LTP protocol only after prior paired conditioning.
Figure 4: LTP produces conditioned response only after prior paired conditioning; protocol two.
A naïve group of animals (N = 5) were tested for conditioned responses to 10 Hz ODI stimulation (shaded blue area) one day after LTP protocol (a), one day after subsequent paired ODI and shock conditioning (b), one day after subsequent LTD protocol (c) and one day after subsequent LTP protocol (d). e, Graph of normalized lever presses for first minute of 10 Hz ODI stimulation one day following indicated protocols. Note that conditioned response is seen following LTP protocol only after prior paired conditioning.
Figure 5: In vivo electrophysiological responses to 10Hz, LTD and LTP protocols.

a, Left, in vivo field response obtained through glass electrode placed in lateral amygdala to single optical stimulus delivered through optic fiber placed 500 μm above electrode tip before (black) and after (red) 10 Hz conditioning protocol. Plot of individual experiment (middle) or average of 10 experiments (right) of field EPSP amplitude (normalized to baseline period) before and after 2 minute 10 Hz stimulation optical stimulation (where indicated). b, Left, in vivo field response (as in a) before (black) and after (red) LTD conditioning protocol delivered through optic fiber. Plot of individual experiment (middle) or average of 10 experiments (right) of field EPSP amplitude (normalized to baseline period) before and after LTD protocol (where indicated). c, Left, in vivo field response (as in a) before (black) and after (red) LTP conditioning protocol delivered through optic fiber. Plot of individual experiment (middle) or average of 10 experiments (right) of field EPSP amplitude (normalized to baseline period) before and after LTP protocol (where indicated). NS indicates non significance, while < 0.01 indicates significance level, paired Student’s t-test, comparing average value at 42-62 minutes with average value at 0-20 minutes.
Figure 6: LTD protocol of ODI significantly reduces auditory fear conditioning; LTP does not reverse auditory extinction.

Animals (N =5) were exposed to auditory fear conditioning (a) or auditory fear conditioning followed by LTD protocol to ODI (b), and subsequently tested for conditioned response. Animals shown in (b) were then exposed to auditory fear conditioning and tested for conditioned responses (c). d, LTD of ODI significantly reduces auditory fear conditioning. Animals shown in (c) were exposed to auditory extinction protocol (see methods) and tested for conditioned responses (e); they were then exposes to LTP of ODI and tested for conditioned response (f). g, LTP of ODI did not reverse extinction of auditory fear conditioning.
Figure 7: Freezing correlates well with reduction in lever presses to previously learned task.
Plot of percent freezing versus reduction in lever presses to previously learned task. Best-fit line indicates significant positive correlation ($R^2 = 0.4$; $p < 0.01$). Data includes results from 3 manipulations (paired ODI conditioning, ODI LTD, ODI LTP). The change in lever presses to previously learned task ($60 \pm 9$) was greater than freezing ($20 \pm 5$; $N = 21$; $p < 0.001$, paired Students t-test).

Figure 8: Expression of oChIEF in auditory regions reaches lateral amygdala.
Diagram (left) and epifluorescent image (right) of coronal section of rat brain indicating areas expressing AAV-oChIEF-tdTomato 2-3 weeks after in vivo injection in auditory cortex (a) and medial geniculate nucleus (b). Note axonal expression of AAV-oChIEF-tdTomato in amygdala (c). Scale bar, 500 μm.
Figure 9: 10 Hz test protocol does not produce conditioned response. Test for conditioned response in naïve animals (N=8), as measured by changes in lever presses normalized to baseline period. Blue area indicates delivery of 10 Hz optical stimulation of the ODI. Each point represents data collected over 1 minute. Error bars indicate SEM. NS indicates non-significant difference, paired Student’s t-test.

Figure 10: Optic fiber locations in different rats used in the behavioral assays. Histologically verified optic fiber tips for rats that responded (blue circles; upper panel is one example) or did not respond (orange circles; lower panel is one example) to optical conditioning. The arrow on the panels shows the location of the tip of optic fiber. Lateral amygdala is indicated by dashed line. Scale bar, 500 μm. Note that ventricle opened during sectioning in the lower image.
Figure 11: Systemic NMDA receptor blockade during conditioning blocks ODI-induced conditioned response.

Animals (N = 5) were injected with MK801 (see methods) and given 10 Hz ODI paired with foot shock and subsequently tested one day later for conditioned response with a 10 Hz ODI test stimulus (a). The same group of animals was then given 10 Hz ODI paired with foot shock (in the absence of MK801) and subsequently tested one day later for conditioned response with a 10 Hz ODI test stimulus (b). c, MK801 significantly blocked conditioning.
Figure 12: Effects of LTD and LTP are rapid and long-lasting. Animals (n=5) were tested for conditioned response one day following pairing of ODI with shock (a). Within one hour of testing animals underwent LTD and were tested for conditioned response 20 minutes later (b). Three days later animals were tested again for conditioned response (c). Following day three testing, animals underwent LTP and were tested for conditioned response 20 minutes later (d). Three days after LTP animals were again tested for conditioned response (e). f, Graph of normalized lever presses for first minute of 10 Hz ODI stimulation one day following indicated protocols.
Figure 13: Optic fiber locations in different rats used in the in vivo recordings.
Histologically verified optic fiber tips (blue circle) and a representative (right panel) image. The arrow on the panel shows the location of the tip of optic fiber. Lateral amygdala is indicated by dashed line. Scale bar, 500 μm. Note different scale bars for diagram and fluorescent images.

Figure 14: Optically driven in vivo and in vitro stimuli produce similar electrophysiological responses.
In both cases, animals were injected in vivo with AAV-oChIEF-tdTomato in auditory regions. Left, in vivo electrophysiological response obtained from glass electrode placed in lateral amygdala and evoked by light pulse delivered through fiber optic cable placed 500 μm above tip of glass electrode. Right, in vitro brain slice electrophysiological response obtained from glass electrode placed in lateral amygdala and evoked by light pulse delivered through fiber optic cable placed above the brain slice. Black trace is before and red trace after bath application of 10 μM NBQX.
Figure 15: LTD reverses LTP and LTP reverses LTD, of in vivo ODI in amygdala.

a, plot of baseline normalized fEPSP in vivo responses to ODI test stimulation (N = 5) following LTP (100 Hz) and LTD (1 Hz). b, same as a for a separate group of recordings (N = 5) following LTD (1 Hz) and LTP (100 Hz).
Figure 16: LTP does not reverse ODI extinction.
a, Animals (N=5) underwent ODI fear conditioning (see Methods) and were subsequently tested for a conditioned response to ODI 24 hrs later. b, Results of ODI conditioned response testing of animals from a following extinction protocol, consisting of 5-7 consecutive days of testing (2 min. of 10Hz light exposure, see Methods). c, Animals from b were administered LTP to ODI and were subsequently tested for conditioned response to ODI 24 hrs later. d, LTP did not reverse extinction of ODI fear conditioning.

All figures, figure legends, and methods and materials, in full, as well as the results and discussion sections, in part, have been submitted for publication of the material as it may appear in Nature, 2014, Sadegh Nabavi, Rocky Fox, Christophe D. Proulx, John Y. Lin, Roger Y. Tsien, and Roberto Malinow, Nature Publishing Group, 2014. The thesis author is co-first author (along with Dr. Sadegh Nabavi) of this paper.
III:

Discussion
In this study we have examined the role of bidirectional plasticity in memory formation. We have demonstrated that optogenetic activation of auditory inputs to the lateral amygdala when temporally paired with shock is sufficient for the formation of associative memory, and that potentiation of synapses between auditory inputs and lateral amygdala neurons occurs during this memory formation. Following memory formation, LTD removes the memory (conditioned response) demonstrated during optogenetic auditory input activation, which can then be reinstated by LTP. We have also shown that LTP at auditory inputs to the lateral amygdala is not sufficient to drive a conditioned response in naïve animals. Additionally, LTP proved to be insufficient to reinstate the associative fear memory in animals that have undergone extinction.

By demonstrating that the pairing of ODI stimulation with shock creates an associative memory that can be reinstated by LTP following removal by LTD, we have provided direct evidence that LTP is necessary for the formation of memories, and that LTD at the synapses potentiated during memory formation is sufficient for memory disassembly.

The fact that LTP of auditory inputs to the lateral amygdala without prior conditioning was unable to produce a later conditioned response demonstrates that potentiation of auditory inputs to the lateral amygdala is insufficient for fear conditioning. This suggests that modification of synapses outside the lateral amygdala, perhaps at somatostatin-positive neurons in the lateral subdivision of the central amygdala, is also necessary for memory formation\textsuperscript{19}. Interestingly,
the inability of LTP to reinstate memory following extinction suggests that a modulation of synapses outside the lateral amygdala also takes place during memory extinction. This lends further support to the idea that extinction is not merely a de-potentiation of synapses, at least not in the lateral amygdala alone.

While previous studies have assessed the relationship between plasticity and memory indirectly using pharmacology\textsuperscript{20} or genetic manipulation\textsuperscript{21,22}, these studies did not provide causal evidence of the effects of changes in synaptic plasticity on memory directly. Our results provide further support to the long-standing view that LTP is necessary for the formation of memories\textsuperscript{1}. In doing so we have demonstrated how optogenetic manipulation can be used not only to evaluate the role of neuronal assemblies in memory, which has been recently shown\textsuperscript{23}, but also how it can be used to induce plasticity and evaluate its effects on behavior.

All figures, figure legends, and methods and materials, in full, as well as the results and discussion sections, in part, have been submitted for publication of the material as it may appear in Nature, 2014, Sadegh Nabavi, Rocky Fox, Christophe D. Proulx, John Y. Lin, Roger Y. Tsien, and Roberto Malinow, Nature Publishing Group, 2014. The thesis author is co-first author (along with Dr. Sadegh Nabavi) of this paper.
IV:

Materials and Methods
Subject. Male Sparague-Dawley rats, age 6-8 weeks for virus injection and
cannula placement and 10-12 weeks for behavioral and electrophysiological
studies, were housed two per cage and kept on a 12/12 hours light-dark cycle
(lights on/off at 7 a.m./7 p.m.). The behavioral studies were done during daylight.
All procedures involving animals were approved by the Institutional Animal Care
and Use Committees of the University of California, San Diego.

Virus. We used a ChR variant, named oChIEF, which is a mammalian codon
optimized version of ChIEF\textsuperscript{15} with the same properties except that it has stronger
expression in mammalian cells and has an additional N-terminal amino acid
residue. Expression was driven by the neuron-specific synapsin promoter\textsuperscript{24}.

Surgery. Male Sparague-Dawley rats, age 6-8 weeks, were anesthetized with
isoflurane for stereotaxic injection of AAV-oChIEF into the medial geniculate
corpus (AP: -5.1 mm and -5.7 mm; ML: 2.9 mm; DV: -5.5 to -6.7 mm) and the
auditory cortex (AP: -5.7 mm; ML: 4.8 mm with a 20° angle; DV: -4.5 to -5.7 mm).
0.4-0.5 µl of virus was injected over a 10-15 minute period. At the end of
injection, pipet remained at the site for 5 minutes to allow for diffusion of the
virus. An optic fiber cannula (Doric Lenses) was implanted just above the dorsal
tip of the lateral amygdala (AP: -3.3 to -3.5 mm; ML: 4.2 mm; DV: -7 mm with a 7°
angle) and secured to the skull with screws and dental cement. Rats were
injected with 5mg/kg carprofen (NSAID) after surgery.
Excitotoxic Lesion. Rats, age 6-8 weeks, were anesthetized with isoflurane for stereotaxic injection of N-methyl-D-aspartate (NMDA) into one amygdala (AP: -3 mm; ML: 4.2 mm; DV: -7-8 mm with a 7° angle). 0.5 µl of NMDA (20mg/ml) was injected over a 10-15 minute period. At the end of injection, pipet remained at the site for 5 minutes to allow for diffusion of the solution.

Behavioral assays

Training. Rats were trained to associate lever press for a reward (40 µl of 10% sucrose per lever press). During the training period rats were kept on a restricted water schedule (2 hours daily of water ad libitum). Training context was a modular operant test chamber (12.5×10×13 in.; context A) with a stainless grid floor and open roof located in a sound attenuating cubicle (Med Associates, St. Albans, VT). The test chamber was equipped with a retractable response lever, a liquid dispenser receptacle and a light above the dispenser that signaled when liquid was injected into the dispenser. The consumption of liquid was detected by a head entry detector in the receptacle; each successive liquid reward was subsequently followed with a 15 second delay after head removal from the receptacle. The system was controlled and the data collected through a MED-SYST-16 interface, which was controlled by MED-PCR IV software running on a PC. Rats were initially trained to associate the reward with the light above the
dispenser receptacle. In a 45-minute session, rats with at least 60 head entries into the receptacle were selected for lever press training.

Lever-press training was conducted in the same context as above, but this time rats had to press a lever to receive the liquid. The level press turned the light above the receptacle on, which in the previous training session they had associated with liquid in the receptacle. Rats with a minimum of 6 responses/min in the first 10 minute of the training session were selected for conditioning.

**Tone conditioning.** The conditioning chamber was a box (12×10.5×13 in.; context B) with an electrified grid floor (Coulbourn Instruments, Allentown, PA) within a larger sound-attenuating box. Rats had full access to water 24 hours before conditioning. Conditioning protocol consisted of 10 trials of 20 second tone (tone volume 80 dB), with randomized intervals (average interval duration 3 minutes). In the paired group tones were co-terminated with a 0.5 second 0.5 mA foot-shock (or one 20 second tone and shock of 0.5 mA for 1 second for mild conditioning$^5$ (Fig. 6)). In unpaired group tones and shocks were separated by at least 1 min$^5$. Paired and unpaired groups received equal number of tones (CS) and shocks (US) in the same context; however, only in the paired group did tone and shock coincide to reinforce the association between CS and US. The next day conditioned rats were placed into the test chamber to measure the effect of CS on their lever presses (for details, see the section for Testing).
**Optical conditioning.** Rats were placed into the conditioning chamber and were attached to an optic fiber patch cord connected to a 473 nm solid-state laser diode (OEM Laser Systems) with 15-20 mW of output from the 200 μm fiber. They were allowed to explore the chamber for 3 min prior to the conditioning. Optical conditioning was 10-trains of blue light (10 pulses of 10 Hz, 2 ms duration) applied at randomized intervals with an average of 3 minute apart. For paired conditioning, the light stimulus co-terminated with 0.5 s of 0.5 mA foot-shock; in unpaired conditioning, the light and shock were separated by a minimum of 60 s. The delivery of shock and light were controlled by a pulse generator (Master-8; AMPI, Jerusalem, Israel). After the conditioning rats remained in the box for additional three minutes before returning to their home cage.

**Testing.** After the conditioning, rats were water restricted for 24 hours before they were tested for lever press. Testing was done in the same context as training except that the floor was a plastic sheet with white and red strips (context C). Testing was a 7-minute session in which rats had to press a lever to receive the liquid (10% sucrose). Rats were attached to the optic fiber patch cord, placed into the chamber, and allowed to explore the environment for 5 minutes before having access to the lever. The testing session, where rats had free access to the lever, was a 3 minute period of no light, followed by two minutes of light on (10 Hz of pulses with 2 ms duration), and two minutes of no light. At the end of
the session rats were returned to their home cage. Only rats that in two consecutive days showed consistent reduction (>30%) in the lever press during the light-on period were used for further behavioral phases. Those who failed the test were examined histologically to locate the position of cannula and viral injection (Figure S3).

Tone-conditioned rats were tested in the same way except that they received 2 minutes of tone instead of light stimulation.

**LTD induction.** Within one hour following testing, rats were placed in a separate context, a translucent plastic container (22.5×15×12 in.; context D), attached to the optic fiber patch cord and allowed to explore the environment for 3 minutes before LTD induction. Optical LTD was induced with 900 pulses of light, each 2 ms, at 1 Hz. After the induction rats remained in the chamber for 3 additional minutes before returning to their home cage. 24 hours after LTD induction rats were tested for the effect of light stimulation on their lever presses.

**LTP induction.** Within one hour following testing, rats were placed in a separate context, a cardboard box (20.5×15.5×14.5 in.; context e), attached to the optic fiber patch cord and allowed to explore the environment for 3 minutes before LTP induction. Optical LTP was induced with 5 trains of light (each train 100 pulses, 100 Hz) at 3-minute inter-train intervals. After the induction rats remained in the
chamber for 3 additional minutes before returning to their home cage. 24 hours after the LTP induction rats were tested for the effect of light stimulation on their lever presses.

During all behavioral assays the light intensity remained the same for each animal. At the end of the experiment, animals were perfused and the location of the optic fiber was verified.

Systemic injection of MK801. Rats were anesthetized with isoflurane for 5 minutes before being given an intraperitoneal injection of MK801 (0.2 mg/kg) in sterile saline. Conditioning protocol was administered 30 minutes following injection.

Perfusion, slicing and imaging. Prior to perfusion, rats were administered a ketamine/dexdomitor (75 and 5 mg/kg respectively) mixture intraperitoneal injection. Rats were then transcardially perfused with ~150 mL of saline followed by ~150 mL of 4% paraformaldehyde in 0.1M phosphate buffer solution (PB, pH 7.4). Brains were then fixed overnight in the same solution and rinsed and stored in 0.1M PB for slicing.

Brains were sliced coronally in 150 μm sections using a vibratome sectioning system and stored in PB. Slices were imaged using an Olympus MVX10 epifluorescent microscope to verify AAV-oChIEF-tdTomato expression in the
MGN, auditory cortex, and their projections to the dorsal lateral amygdala. Additionally, appropriate positioning of the optic fiber cannula over the lateral amygdala was verified.

**In vitro recording.** For extracellular field potential recording acute slices (as described in5) were prepared from 3-4 month old rats expressing AAV-oChIEF in the medial geniculate nucleus /and or auditory cortex. Extracellular field potentials were recorded with Axopatch-1D amplifiers (Axon Instruments) in dorsal tip of the lateral amygdala with glass electrodes (1–2 MΩ) filled with the perfusion solution. The auditory projection to the lateral amygdala was evoked by optical stimulation above the recording site. To measure AMPA-R field potential 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) (10 μM) was added at the end of the experiments. Data were acquired and analyzed using custom software written in Igor Pro (Wavemetrics). Perfusion solution contained: 119 mM NaCl, 2.5 mM KCl, 4 mM CaCl2, 4 mM MgCl2, 26 mM NaHCO3, 1 mM NaH2PO4, 11 mM glucose (pH 7.4), and gassed with 5% CO2/95% O2.

For whole cell recording, acute slices (as described in27) were prepared from 3-4 month old rats expressing AAV-oChIEF in the medial geniculate nucleus and/or auditory cortex. Whole-cell recordings were obtained from individual cells in dorsal tip of LA using glass pipettes (3–4 MΩ) filled with internal solution containing, in mM, cesium methanesulfonate 115, CsCl 20, HEPES 10, MgCl2
2.5, Na\textsubscript{2}ATP 4, Na\textsubscript{3}GTP 0.4, sodium phosphocreatine 10, and EGTA 0.6, at pH 7.25. External perfusion consisted of artificial cerebrospinal fluid (ACSF), containing 119 mM NaCl, 2.5 mM KCl, 26 mM NaHCO\textsubscript{3}, 1 mM NaH\textsubscript{2}PO\textsubscript{4}, 11 mM glucose, supplemented with 1 mM MgCl\textsubscript{2}, and 2 mM CaCl\textsubscript{2}, 100 μM picrotoxin and 1 mM Sodium L-ascorbate. Synaptic responses were evoked every 10 seconds by stimulating auditory projections to the LA using 2ms of blue light generated by the epifluorescece microscope and passed through the 60X objective lenses placed immediately above the recorded cell. AMPA:NMDA ratio was calculated as the ratio of peak current at -60mV to the current at +40mV, 50 ms after stimulus; both values substracted from the current at 0mV.

**In vivo recording.** Four weeks after injection of AAV-oChIEF-tdTomato into auditory regions (8 animals were injected in both MGN and auditory cortex; 2 animals were injected in only auditory cortex; results were pooled), rats were anesthetized with a set of three injections of 700 μl urethane (330 mg/ml) given at 10 minute intervals 2 hours before the recording\textsuperscript{28} and then mounted on a custom made stereotaxic frame with an adjustable angle, to hold the head in a fixed position during the recording. The body temperature was regulated by a heating pad. Using aseptic surgical tools the skull was exposed and a hole (~3 mm) was made, centered at -3.3mm AP and 4.2mm ML. The recording electrode was a glass pipet (4-5mΩ) filled with 0.9% NaCl. The recording electrode was connected to a Axopatch-1D amplifier. The signal was amplified (*1000), filtered
(0.1–500 Hz) and digitized at 10 kHz using an Instrutech A/D interface. Data were acquired and analyzed using custom software written in Igor Pro (Wavemetrics).

For optical stimulation, the optic fiber was glued to the glass pipet so that the tip of the fiber was 500 μm above the tip of the glass pipet to form an optrode. The optic fiber was connected to a 473 nm solid-state laser diode (OEM Laser Systems). The parameters for the optical stimulation were identical to those used during behavior (2 ms duration, 15-20 mW intensity). The optrode was slowly lowered in at a 7° angle following the start of stimulation. After establishing a stable baseline of at least 30 minutes (stimulation frequency 0.033Hz) at the recording site (DV: -7 to 7.5), 2 minutes of 10 Hz stimulation was evoked, which was followed by 40 minutes of 0.033 Hz stimulation. Subsequent LTD and LTP, with the same parameters used in the behavioral assay, were induced 40 minutes apart. Electrode resistance and light intensity were monitored before and immediately after the recordings to ensure that there was no change during the course of recording. All animals were perfused after the recordings and the position of the recording site verified.

**Analysis.** The number of lever presses were binned for each minute and normalized to the 2-minute period before light stimulation. Suppression ratio was measured by dividing the number of lever press during the first minute of
conditioning stimulus (tone or optical stimulation) by that immediately preceding the stimulus.

To minimize the voltage dependent conductance component, the initial slope of field excitatory postsynaptic potentials were measured using a custom written MatLab program.

Excitatory postsynaptic current amplitude was measured by averaging a fixed 3-ms window covering the peak amplitude and subtracting from an average current window before stimulation.

All values given in the text and figures indicate mean ± SEM. Student’s paired and non-paired t-tests were used with p < 0.05 considered as significant. All behavioral were also analyzed with Wilcoxon rank-sum test (Matlab statistic toolbox) and yielded the same significance values.

All figures, figure legends, and methods and materials, in full, as well as the results and discussion sections, in part, have been submitted for publication of the material as it may appear in Nature, 2014, Sadegh Nabavi, Rocky Fox, Christophe D. Proulx, John Y. Lin, Roger Y. Tsien, and Roberto Malinow, Nature Publishing Group, 2014. The thesis author is co-first author (along with Dr. Sadegh Nabavi) of this paper.
References


