# The Role of 40hz Oscillation at Basolateral Amygdala in Mediating the Retrieval of Extinction Memory

An Honors Thesis for the Department of Psychology

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**Abstract**

While exposure therapy is the standard of care for post-traumatic stress disorder, it rarely leads to a full recovery. Thus, it is imperative to investigate the biological mechanisms underlying fear extinction to understand the power and limitations of exposure therapy. During extinction learning, an analogous process of exposure therapy in animals, a new extinction/safety memory is formed and competes with the original fear memory for retrieval. The current study aimed to probe the role of gamma range (30 -100+ Hz) oscillation at the basolateral amygdala (BLA), a crucial brain region for valanced memory learning in supporting such competition. Unpublished data in the Reijmers Lab suggested the power of 40 Hz oscillations at BLA was increased during extinction learning and retrieval of the extinction memory. This suggests a role of BLA 40 Hz in biasing the retrieval of extinction over fear memory. To explore this hypothesis, the fear level, as quantified by the freezing level of mice, was measured during exogenously induced 40 Hz oscillation by optogenetic manipulation of Parvalbumin (PV) interneurons at the BLA. The level of fear reduction at 40Hz was further compared with reduction induced by different frequencies of stimulation and at different memory states to determine whether 40 Hz oscillation uniquely in the gamma range bias the retrieval of extinction memory. However, even after accounting for the extinction learning difference in individuals, results showed that 40 Hz failed to induce significant fear reduction compared to other stimulation conditions. The insignificant effect of 40 Hz oscillation observed in the current data was most likely due to a lack of subjects. The inclusion of more individuals is necessary to truly determine the role of BLA 40Hz oscillation in extinction learning.

*Keywords*: optogenetic, PTSD, extinction learning, gamma oscillations, exposure therapy

## Introduction

Post-traumatic disorder (PTSD) has a lifetime prevalence of 7-8% in the United States. Kearns et al., 2012; Sareen, 2014) While currently available medications and therapies can reduce symptoms, there is a need for more effective treatments. Exposure therapy is the standard of care in treating PTSD and other fear disorders. In exposure therapy, a safe space is created where individuals with PTSD could be "exposed" to the items and/or environments they fear and wish to avoid. Such exposure helps reduce fear and decrease avoidance. However, exposure therapy rarely leads to a full recovery from or the erasure of the maladaptive fear. Evidence from the past few decades from both human studies and extinction learning, an analogous process of exposure therapy in animals, is supportive of the theory that instead of eliminating the fear memory, extinction learning/exposure therapy creates a distinct safety memory that competes with the original fear memory for expression (Myers & Davis, 2017; Orsini & Maren, 2012). This theory is most strongly supported by recent evidence showing fear and extinction/safety memory have separate engrams, neuronal ensembles that hold the specific memories, and both engrams are present in the basolateral amygdala (BLA) (Zhang et al. 2020). Thus, to better understand the reasons behind the power and limitations of exposure therapy, it is important to investigate the biological mechanisms at BLA that support the acquisition of extinction and fear memory and the processes that determine whether extinction or fear memory is retrieved and expressed.

Circuit oscillations in the BLA are of particular interest to valanced memory learning (Bocchio et al. 2017; Headley & Weinberge, 2013; Headley et al. 2021). It is widely observed that learning induces changes in circuit oscillations, which is thought to reflect the degree of synchrony of cellular and synaptic activities of neurons in a circuit (Headley & Pare, 2013;

Tovote et al., 2015). Circuit oscillations are known to recruit neuronal ensembles at different frequencies and to induce spiking resonance from neurons within, enabling them to be more receptive and responsive to synaptic inputs occurring at the same frequency. All these properties of circuit oscillation make it a prime candidate for unraveling the biological mechanisms behind the acquisition and retrieval of fear and extinction memory.

Accumulating evidence in the past decades suggests a crucial role for gamma range oscillations at the BLA in valanced memory (Bocchio et al. 2017; Headley & Weinberge, 2013; Headley et al. 2021). BLA, as a subnucleus of the amygdala, has a microcircuitry more conducive to gamma generation than the lateral amygdala subnucleus (Headley et al., 2021). This is likely due to its higher incidence of fast-spiking interneurons (FSI), which are mostly PV interneurons, and denser interconnections between reciprocally connected excitatory principal neurons (PN) and FSIs These specific mechanisms of BLA micro-circuits could explain the observation of spontaneous and intrinsic gamma oscillation in BLA in absence of stimuli and other gamma input from outside regions. Many previous optogenetics studies have also successfully induced gamma rhythm at the BLA by stimulating PV-interneurons with ChR expression at gamma frequencies. (Headley et al. 2021; Ozawa et al. 2020; Stujenske et al, 2014)

Furthermore, gamma oscillation power at the BLA is not only increased during valanced situations but also during neutral stimuli that come to associate with emotional events through learning, affirming the role of gamma in valanced memory acquisition (Headley & Pare, 2013). Gamma oscillation at BLA could potentially fulfill this role by modulating the efficacy of excitatory synaptic transmission between PNs and between PNs and FSIs (Headley et al., 2021). In addition, high gamma oscillations at BLA, more so than all other rhythms, synchronize PNs. (Amir et al, 2018) The increase in synchrony would also enable gamma to better recruit circuits

in brain regions downstream of the PNs projections which are important in the acquisition, consolidation, and retrieval of valanced memory (Bocchio et al. 2017).

These effects of gamma could be partially responsible for establishing the memory engrams, specific to either fear or extinction memory, and reactivation of oscillations in the gamma frequency could potentially reactivate these engrams. In fact, Dr. Ni, a former postdoc of the Reijmers lab has recently found that the power of 40Hz oscillation is increased during extinction learning and retrieval of extinction memory (unpublished data). It is possible that 40Hz oscillation helps recruit neurons to the extinction engrams during extinction learning and reactive these extinction engrams during retrieval. Evidence supporting this postulation came from past research in the Reijmers lab. In previous studies, extinction learning was shown to increase perisomatic inhibition from PV interneurons (GABAergic) to fear engram neurons (Trouche et al. 2013) Most importantly, chemogenetic silencing of PV interneurons erased the effect of extinction learning and instead promote fear engrams activation and retrieval of fear memory (Davis et al., 2017). Considering the importance of PV interneuron in extinction learning and its role in generating the gamma oscillations at the BLA, it is thus reasonable to propose that 40Hz oscillation facilitates extinction learning and retrieval via the PV-interneurons.

My senior thesis built on and occurred in parallel with Dr. Ni's research and employed a classic contextual fear conditioning and extinction learning paradigm where the freezing level was used as the operational measure for fear level. We hypothesized that after the mice had acquired fear and extinction memory from fear conditioning and extinction learning trials, our optogenetic manipulation of BLA PV-interneurons to induce 40Hz oscillation would bias the retrieval of extinction memory. Experimentally, this would mean the freezing level would be decreased by exogenously induced 40Hz oscillation during the retrieval trial. We predicted that

the freezing reduction effect of 40hz should be memory specific, so 40hz should only elicit the effect in mice who have acquired both fear and extinction memory. We also expected that 40hz, rather than all stimulation frequencies in the gamma range, should uniquely induce freezing reduction. The results of the studies would help us determine the role of gamma range oscillations in the retrieval of contextual extinction memory and inform us of the possible mechanisms mediating the competition between fear and extinction memory.

## Method

### Animals

All animal procedures were performed in accordance with the NIH Health Guide for the Care and Use of Laboratory Animals and were approved by the Tufts University Institutional Animal Care and Use Committee. PV-Cre mice (2–6 months old) used were heterozygous for a PV-IRES-Cre knock-in locus (B6; 129p2-Pvalb$^{tm1(cre)Arbr}$/J). Mice had food and water ad libitum and were socially housed until the implantation surgery with optic fiber cannulae; after which, mice were individually housed in pinnacle cages. Mice were of at least 10 weeks of age before virus injection. All mice were kept on a regular light-dark cycle, and all experiments were performed during the light phase. Both female and male mice were used, and their data were pooled for final analysis.

I completed data collection on 6 mice, but the behavioral data from only one mouse (Subject *7212*) was included in the Results section due to the histology exclusion criteria (see below). An additional 7 mice from Dr. Ni's experiments (a total of 8 mice) were included for the analysis in the Result section. Six out of the eight mice had a complete set of behavioral data and were considered for 2-Way ANOVA analysis.

**Stereotaxic surgery**

Mice were anesthetized with isoflurane and weighted before being fixed onto a stereotaxic apparatus (Kopf) for virus injection. After incision, small holes were drilled into the skull above the left and right BLA (AP − 1.35, ML ± 3.45, DV − 5.15 mm) before virus injection. After injection, the needle was left in place for 10 min before being slowly retracted. The incision was sutured, and mice were weighed and monitored to ensure recovery. For BLA optogenetic stimulation experiments, AAV-Ef1a-DIO-hChR2 (H134R)-mCherry (UNC Vector Core, Karl Deisseroth) was injected into BLA (AP − 1.35, ML ± 3.45, DV − 5.15 mm). All mice in this experiment were injected and targeted bilaterally. Mice were implanted with fiber optic cannulae bilaterally at 3 weeks following virus injection (Thorlabs, CFM12L05) in the BLA (AP − 1.35, ML ± 3.45, DV −5 mm). The cannulae were affixed to the skull with dental cement mixed with black charcoal powder to minimize the dispersion of light. Additional stainless-steel screws were also fixed onto the skull to increase traction for the dental cement.

**Behavioral Experiments**

Behavior experiments started 1 week after the implantation of cannulae, and at least 4 weeks after virus injection. None of the mice had prior procedures or testing performed. Mice underwent three fear conditioning trials (FC1, FC2, and FC3) with 3 h between each trial. The total duration of each training trial was 500 s. The mouse was placed in a square chamber with a grid floor (Context A; Coulbourn Instruments; H10-11RTC). At 240, 300, 360, and 420 s, a foot shock was delivered (2 s and 0.70 mA). On days 2 and 3, mice were subjected to a maximum of two extinction trials per day. The mice must complete 2 extinction trials and complete their extinction training when their freezing level during the first 600 seconds of a trial dropped below 50% or after 4 trials of extinction training. The 50% threshold was chosen to avoid the flooring

effect where the freezing level was too low during the Retrieval A trials to observe a possible effect in freezing reduction by the optic stimulations. Each extinction trial was 1200 s long, with an inter-trial interval of 2 h. (how do we tell enough extinction learning took place?) For each extinction trial, the same box used in fear conditioning (Context A) was used but no foot shocks were delivered. On day 4 of the experiment, mice were submitted to a single 1200s retrieval trial in context A and then immediately underwent a 1200s retrieval trial in context B, which consisted of a rectangular plastic box with bedding sprayed with 10% acetic acid and striped walls.

***In vivo* optical stimulation.**

A 20hz, 40hz, 70hz, and non-stimulation analog sinusoidal stimulation protocol was designed in Matlab software and fed through to a laser (Laserglow, LRS- 0473 DPSS Laser). Laser output at fiber tip at the peak of sine wave 10 mV for all stimulations. Optical stimulation was performed during the first 500s of the first extinction trial, retrieval trial in context A, and retrieval trial in context B. In addition, optical stimulation similar to those performed later in extinction and retrieval trials was performed on each mouse one day before its first fear conditioning trial to habituate the mice to the sudden appearance of light. Four intervals of optic stimulation were performed for each stimulation condition (20hz, 40hz, 70hz, and non-stimulation) with a five-second break between each interval (total intervals = 16). The order of the 16 stimulation intervals was randomized across trials and across animals to ensure the absence of order-dependent effects.

**Quantification of freezing behavior.** Freezing behavior was recorded using a digital camera connected to a computer with Actimetrics FreezeFrame software. Freezing behavior was quantified through the motion index generated by the FreezeFrame software through automated

video analysis. A minimum motion index threshold hold was manually selected based on the distribution of motion index to minimize the inclusion of motion artifact (i.e. movement of optic fiber) as mice movements. Quantification of the freezing behavior of one mouse was also performed manually by the author, and the data were presented separately.

**Histological Analysis.**

Mice were deeply anesthetized and intracardially perfused with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde (PFA 4%) dissolved in 0.1 M PB. Brains were extracted and fixed in PFA 4% for 24 h and then soaked in 30% sucrose for 48–72 h. 30-µm thick coronal sections were obtained with a cryostat. Sections were stored in phosphate buffered saline (PBS) at 4 °C until use Sections were mounted on slides and cover-slipped. A widefileld epifluorescence microscope (Keyence BZ-X700) was used to acquire images to confirm sites of optic fiber cannulae implant and virus expression at the injection site. Images were obtained at 10–20× and stitched together using Keyence software.

**Statistical Analysis.**

Statistical tests were performed using R and Excel. All statistical tests were two-tailed.

**Subject Exclusion Criteria**

Mice were excluded from analysis if they had insufficient ChR2-mCherry expression within BLA and/or if the expression was not targeted to the BLA. Mice were also excluded if the tip of the optic fiber cannulae implants missed the BLA. The exclusion of mice was determined blind to experimental conditions.

## Results

**Histology Results**

The authors independently acquired data from 6 mice. For two of the mice, the optic fiber cannulae failed before the start of the RET_A trial and thus were excluded from analysis since no RET_A data was recorded. Based on histological analysis, another 3 mice were further excluded. Of these 3 mice, one had the correct optic fiber cannulae placement at the BLA, but the virus expression was too low. Another had good virus expression at BLA but cannulae implants missed the BLA. For the last mouse of the 3 mice, the histology was too inconclusive to determine the correct cannulae placement and sufficient virus expression. Only one mouse out of the 6 mice had both good virus expression and cannulae placement. This mouse will be referred to as *7212* for the rest of the paper (Fig 1).

Fig.1a

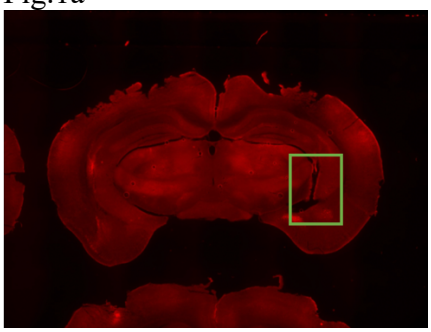

Fig.1b

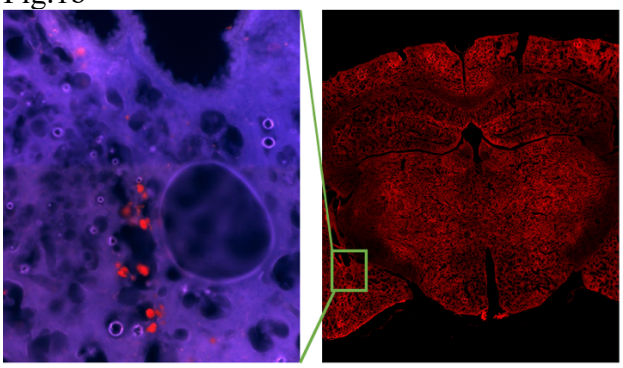

Fig.1c

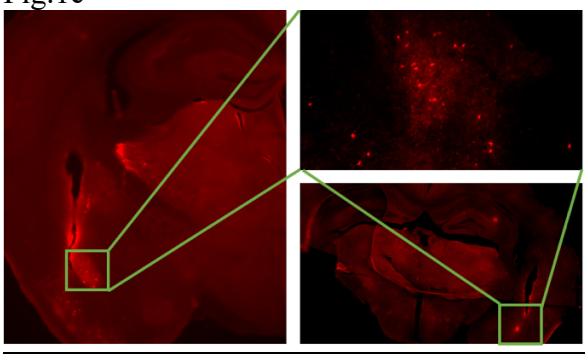

Fig. 1d

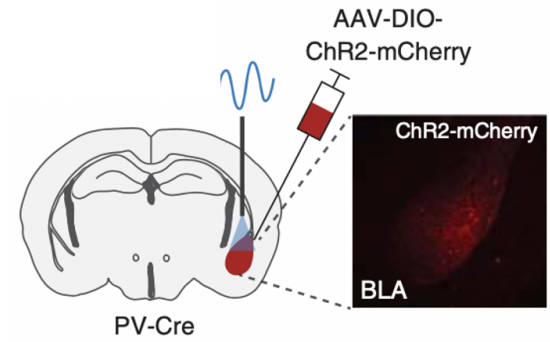


**Fig 1. Histology Examples. a**. Missed Implant. Good virus expression at BLA but implant missed target location. **b**. Low Virus expression. Correct implantation site, but little virus expression. **c.** *7212* Histology. Good virus Expression and Implant Location (virus expression shown as little red dots, implant location shown as black vertical laceration) **d**. surgery schematics

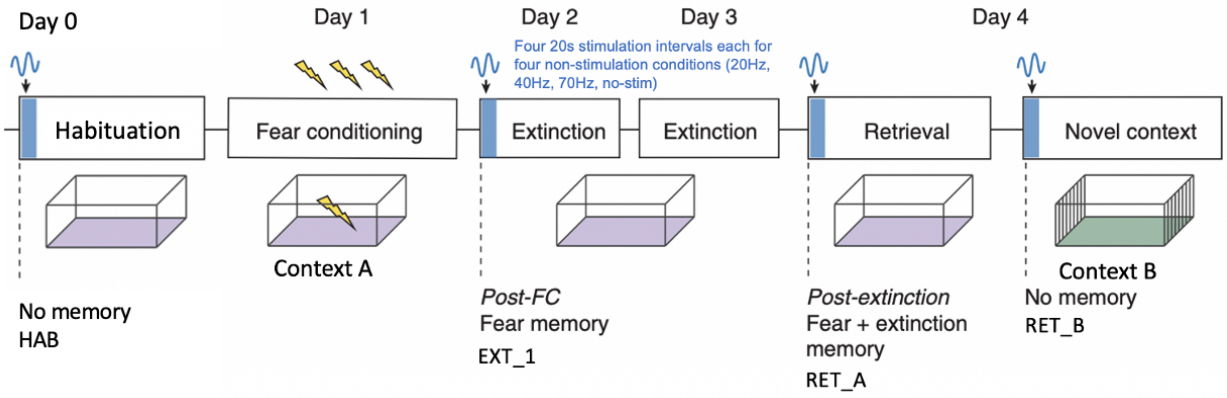


**Fig 2. Schematic of Experimental Design.** see Methods for details

**Successful acquisition of fear and extinction memory in subject *7212***

Mouse subject *7212* with ChR2 in BLA PV-interneurons was subjected to the behavioral paradigm detailed in Fig 2. The four 20s simulation intervals for each of the four stimulation conditions (20Hz, 40Hz, 70Hz, and non-stimulation) were distributed randomly during the first 500s during each of three different trials: EXT_1 (fear memory associated with context A), RET_A (fear + extinction memory associated with context A), and RET_B (un-conditioned context B, no memory associated) (Fig.2). The effects of the identical four stimulation conditions were examined across the three trials each associated with different memories. This was to probe the role of 40Hz stimulation in memory retrieval instead of simple behavior control. If 40Hz oscillation simply recruited networks that support the non-freezing behavior, I would expect to see that 40Hz stimulation reduces freezing in all three trials irrespective of the memories associated. If 40Hz oscillation indeed biases the retrieval of extinction memory over fear memory, one would only observe the effect in the RET_A trial.

Before analyzing the effect of BLA stimulations, however, it was crucial to verify if fear and extinction memories associated with Context A were successfully acquired. The changes in freezing levels across trials in the absence of BLA stimulation were shown in Fig 3. The bar graph shows the freezing level of the mouse during the first 45s of HAB, EXT_1, RET_A, and RET_B trials, as well as the last 70s of all three fear conditioning trials (FC1-3) and the first 600s of EXT_2. These time intervals were chosen to reflect innate freezing level changes as the result of fear and extinction learning and not of stimulation conditions. The freezing level at EXT_1 would only reflect the fear level after the acquisition of fear memory, and the freezing level at RET_A reflect the fear level after acquiring both fear and extinction memory. Since the mice had neither fear nor extinction memory for Context B during RET_B, the freezing level should reflect the mice's natural freezing level when exposed to a new environment.

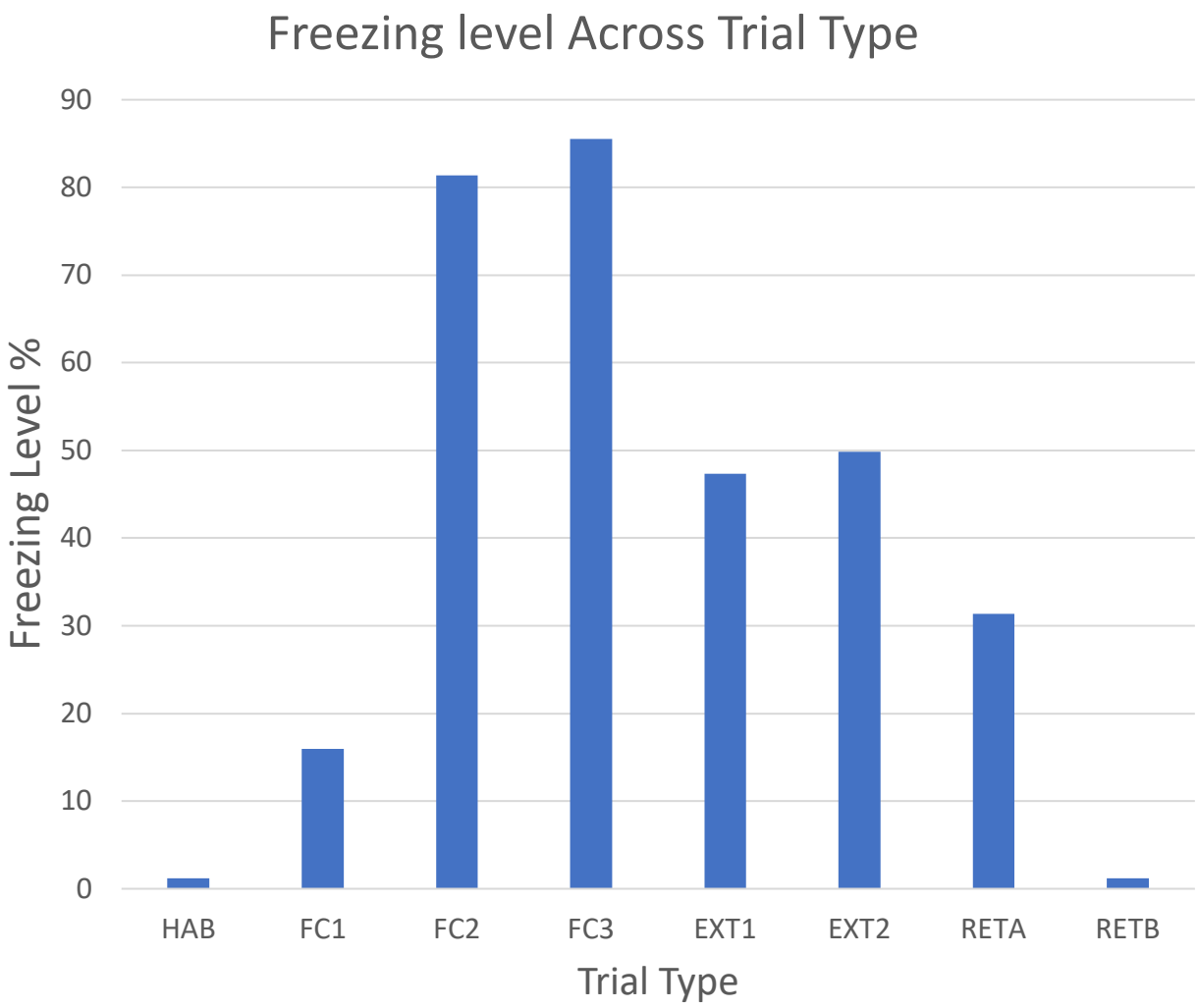


**Fig 3. Freezing Level changes across trial types for *7212***. Fear Conditioning (FC), Habituation (HAB)

As shown in Figure 3, the freezing level, 1.18%, during the first 45s in Retrieval B was comparable to that during the habituation trial which also served as the baseline of the mouse's activity in a new environment with no associated valanced memory. The freezing level increased readily across fear conditioning trials to 85.5 percent which was well above the baseline in HAB. After two trials of extinction learning, the freezing level decreased to 49.88 %, which was around 58% of the peak freezing level in FC3 (a 42% reduction). These data indicated that both

adequate fear and extinction learning occurred in *7212*, and the fear memory acquired was only associated with context A, not B.

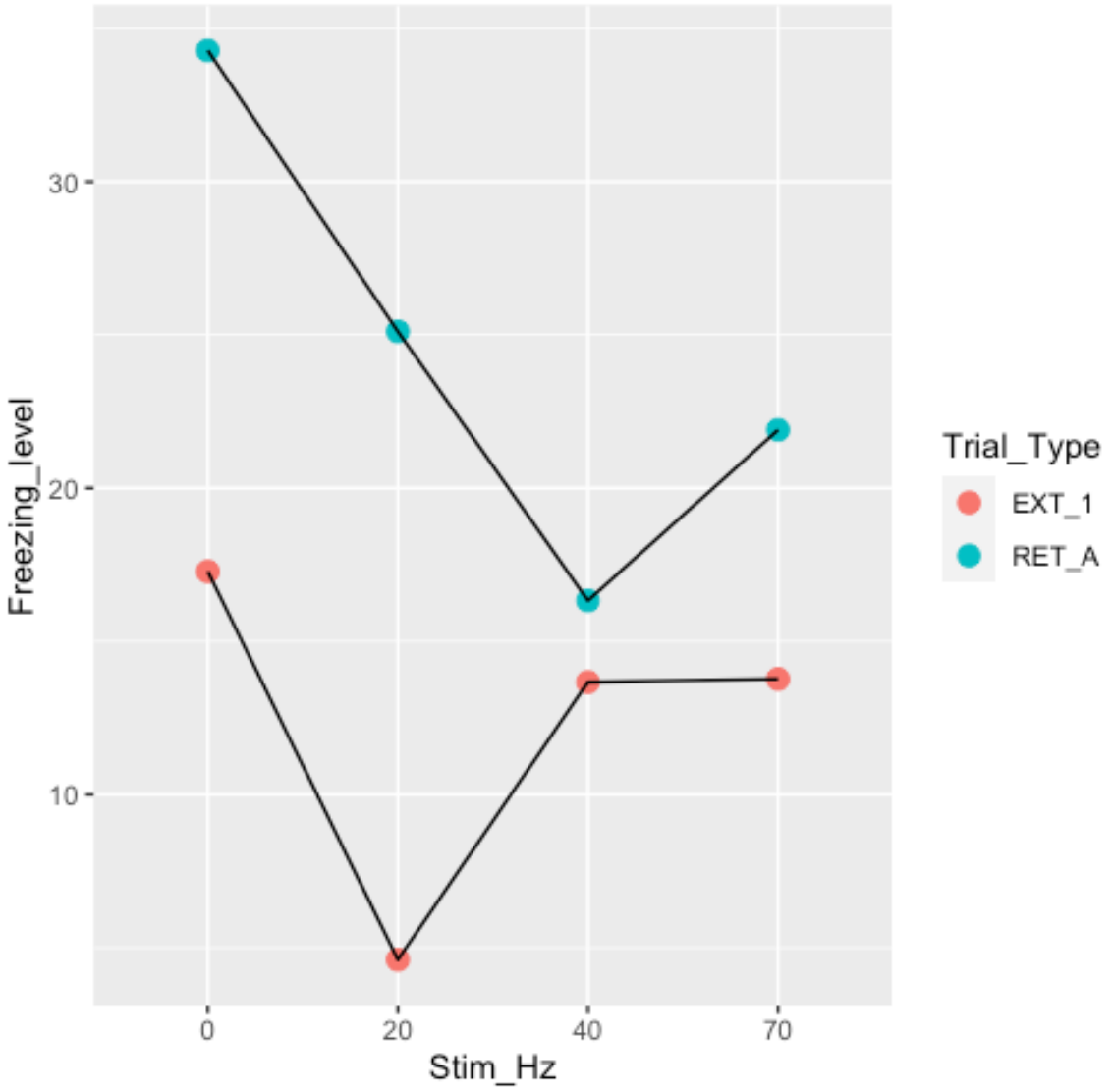


**Fig 4. Average freezing level across different stimulation conditions for different trial types for *7212*.** 0 stimulation condition refers to the non-stimulation condition.

**Possible Effects of 40hz Stimulation Across Trial Type in Comparison with Other Stimulation Conditions**

Unfortunately, due to data corruption, stimulation frequency data in RET_B data for subject *7212* could not be retrieved, so the average freezing level could only be determined for RET_A

and EXT_1 for different stimulation conditions (Figure 4). Interestingly, the average freezing level during periods of non-stimulation was higher for RET_A (after the mouse has completed extinction learning) compared to EXT_1 (when the mouse has yet to acquire any extinction memory). One would expect that without interference from BLA stimulations, periods of non-stimulation would reflect the intrinsic level of freezing that was solely dependent on the degree of fear and extinction learning. Following this logic, the freezing level should be lower in RET_A compared to EXT_1.

Fig. 5a

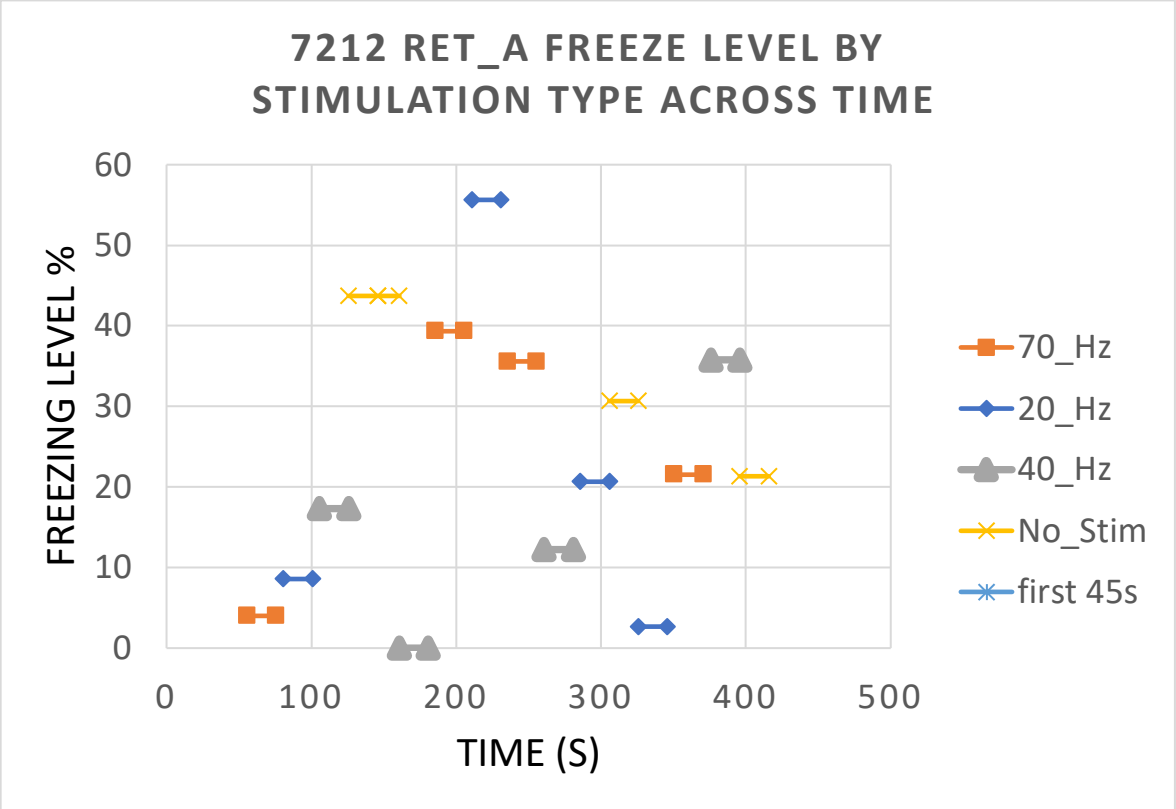

Fig. 5b

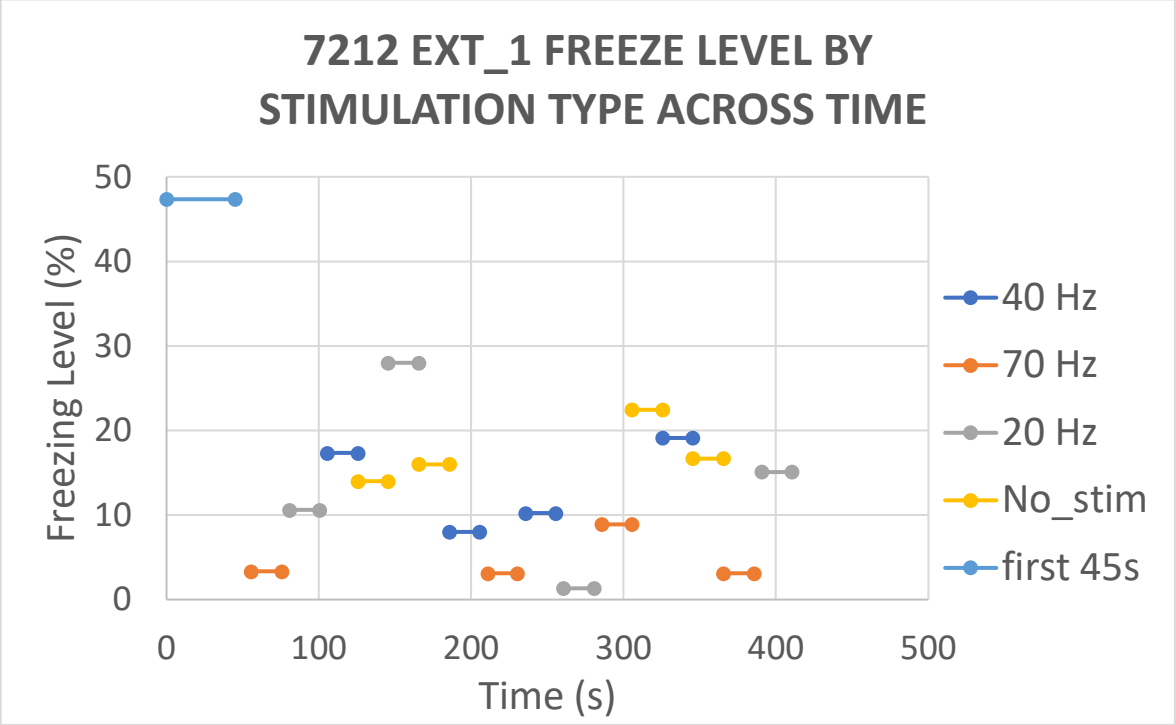


**Fig 5. Freezing level by stimulation condition across time in RET_A and EXT_1 for *7212*.** **a.** freezing level at RET_A, (after the acquisition of fear and extinction memory). **b.** freezing level at EXT_1 (after the acquisition of fear memory only)

To better understand this unexpected observation, freezing levels for the four intervals for each of the four stimulation conditions were organized along the time axis of RET_A and EXT_1 to provide more context (Fig 5 a,b). As shown in Fig 4a,b, average freezing levels during the non-stimulation condition in both trials were not affected by extreme outliers. This called into question the conclusion made in the previous paragraph – if subject *7212* had indeed successfully obtained fear and extinction memory. The author argues that considering non-stimulation intervals occurred in between stimulation intervals, the freezing level obtained could be influenced by some residual effects of stimulation. Consequently, the freezing levels obtained from periods specified for Fig 3 better captured the changes in freezing levels purely due to fear and extinction learning.

**The behavior of *7212* in the larger context**

While the case study of *7212* is interesting, a greater number of subjects was needed to determine whether 40hz stimulation indeed promotes the retrieval of safety/extinction memory. Thus, data from an additional 7 mice were acquired from Dr. Ni, resulting in a total of 8 (7 from Dr. Ni and one, *7212,* from the author) mice used in the summary statistics below.

**Summary statistics of behavior data from 8 mice**

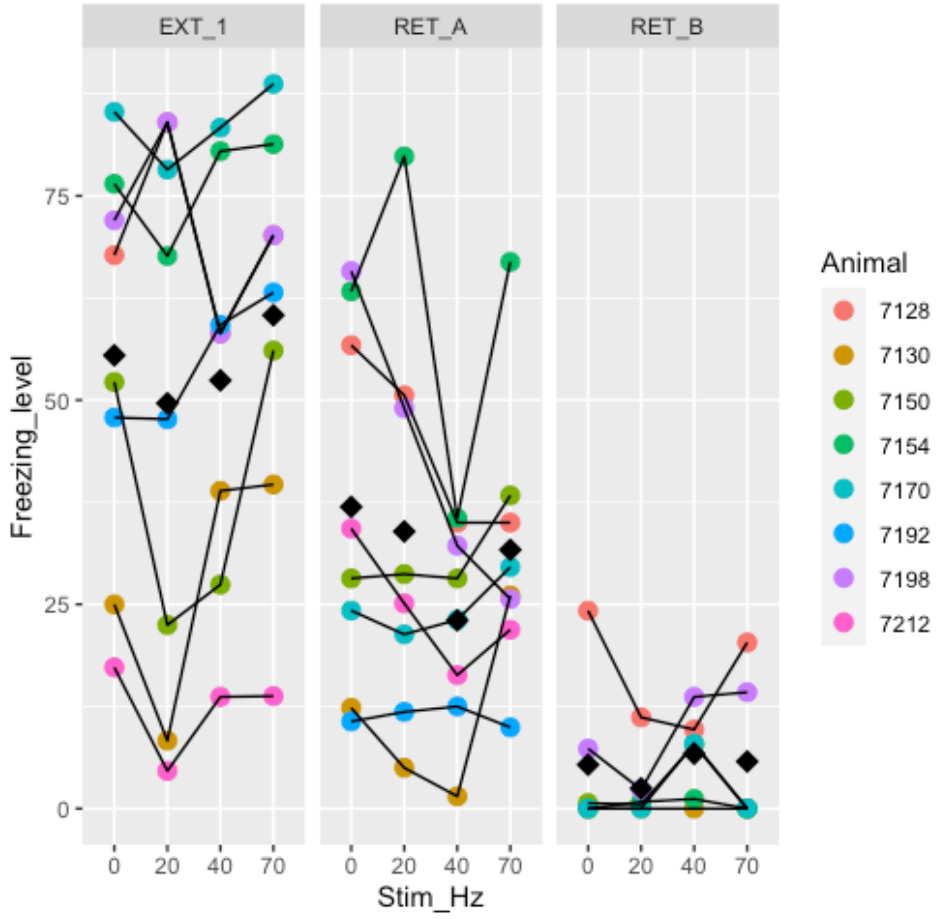


**Fig 6. Averaged Freezing Level Data for each of the 8 mice by trial type and stimulation condition.** Black diamonds indicate means.

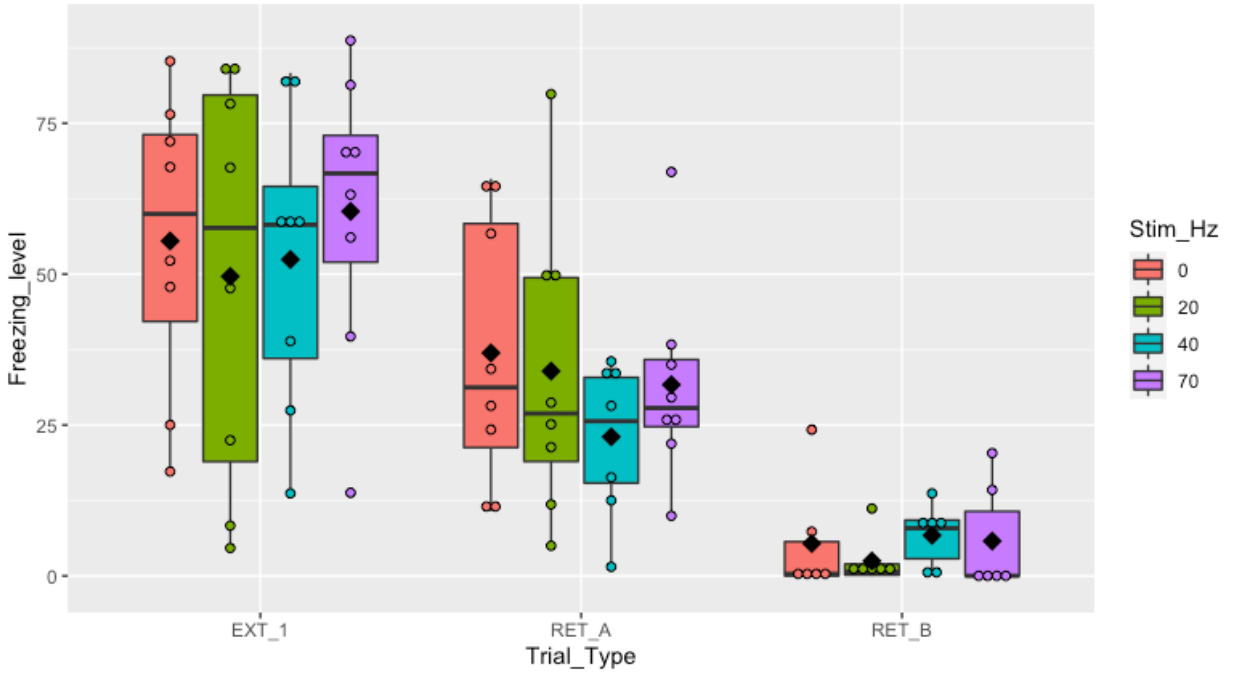


**Fig. 7. Averaged Freezing Level of 8 mice organized into Boxplots**

Averaged freezing level for each stimulation condition across trials EXT_1, RET_A, and RET_B were shown in Fig 6. The same data was organized into a box plot (Fig 7) to identify the presence of extreme outliers defined as data points either above the third quartile + 3xIQR (interquartile range) or below the first quartile - 3xIQR. Using this threshold, the averaged freezing levels of animal *7128* in non-stimulation and 20Hz stimulation conditions during RET_B were considered to be extreme outliers. Shapiro test of normal distribution also identified freezing levels distribution in non-stim, 20Hz, and 70Hz stimulation conditions in RET_B as non-normally distributed. This was probably due to the flooring effect of the freezing level in the unconditioned Context B where the mice were most likely to be actively exploring. Based on the means of freezing levels as presented in Fig 7, 40hz appeared to reduce freezing more than other stimulation conditions. Though a repeated measured 3 by 4 ANOVA test was necessary to verify this trend.

**Freezing levels changed significantly through extinction learning but were not significantly affected by BLA stimulation in different gamma frequencies**

Considering two (*7212, 7192*) out of the 8 mice lacked RET_B data, they were excluded from further analysis. 2 way repeated measured ANOVA test could only be completed for n=6 mice with freezing level data from all three trials (EXT_1, RET_A, RET_B). While stimulation condition x trial type interaction effect failed to reach significant level, $F(6,30)$, $p = 0.24$, the main effect of trial type was significant, $F(2,10)$, $p =< 0.0001$ (Table 1, Fig 8). Post-hoc pair-wise comparison t-test with Bonferroni adjusted $p$ values for Ext_1 vs. Ret_A, Ext_1 vs. Ret_B, Ret_A vs. Ret_B were all significant. (Table 2, Fig.8) In combination with the observed differences in Frz_levels in Fig 7, the data indicated extinction learning significantly reduced expression of fear memory in Context A irrespective of different stimulation conditions. It suggested that as a group, the 6 mice had successfully acquired extinction memory specific to Context A.

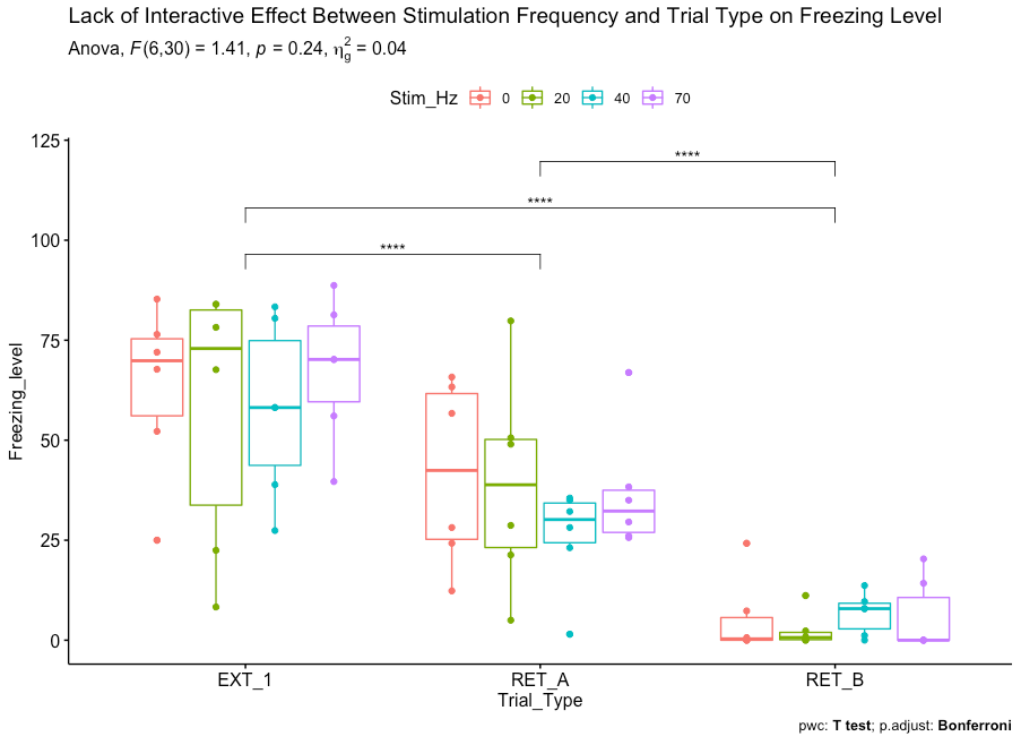

**Fig. 8. Lack of interactive Effect Between Stimulation Conditions and Trial Type on Freezing Level for Mice n=6.** see Table 1,2 for detailed ANOVA and Post-hoc statistics included in this figure. Trial type main effect is significant (see Table 1) and pair-wise comparison T-test with Bonferroni p.adjust was completed for Ext_1 vs. Ret_A, Ext_1 vs. Ret_B, Ret_A vs. Ret, all of which were significant as marked by **** in the figure.

| Effect | DFn | DFd | F | p | p<.05 | ges |
|---|---|---|---|---|---|---|
| Stim_ Condition | 3 | 15 | 2.047 | 0.15 | | 0.026 |
| Trial_Type | 2 | 10 | 27.422 | 8.72E-05 | * | 0.644 |
| Stim_Condition:Trial_Type | 6 | 30 | 1.411 | 0.243 | | 0.036 |

**Table 1. 3 x 4 Repeated Measures ANOVA Statsitics for effects on freezing level in mice n=6**

| Column1 | group1 | group2 | n1 | n2 | statistic | df | p | p.adj | p.adj.signif |
|---|---|---|---|---|---|---|---|---|---|
| Freezing_level | EXT_1 | RET_A | 24 | 24 | 5.849695 | 23 | 5.82E-06 | 1.75E-05 | **** |
| Freezing_level | EXT_1 | RET_B | 24 | 24 | 11.99544 | 23 | 2.23E-11 | 6.69E-11 | **** |
| Freezing_level | RET_A | RET_B | 24 | 24 | 7.61204 | 23 | 9.96E-08 | 2.99E-07 | **** |

**Table 2. Pair-wise comparison T-test for significant Trail Type main effect on freezing level for mice n=6**

However, the lack of stimulation frequency main effect and interactive effect between stimulation frequency and trial type meant that 40hz stimulation did not have the predicted effect on biasing the retrieval of extinction memory over fear memory. If the data were to verify our predictions, we would observe a significant stimulation condition X trial type interaction effect. In the following one-way repeated ANOVA test to understand the effect of stimulation condition for each trial type, we would ideally only observe a significant main effect of stimulation condition during RET_A since this is the only trial where fear and extinction memory would compete for retrieval. The subsequent pair-wise comparison T-test should reveal that freezing level during 40hz stimulation was significantly lower than that during all other stimulation

condition. 40Hz stimulation should not influence freezing level during EXT_1 and RET_B since there was no extinction memory associated with the trials.

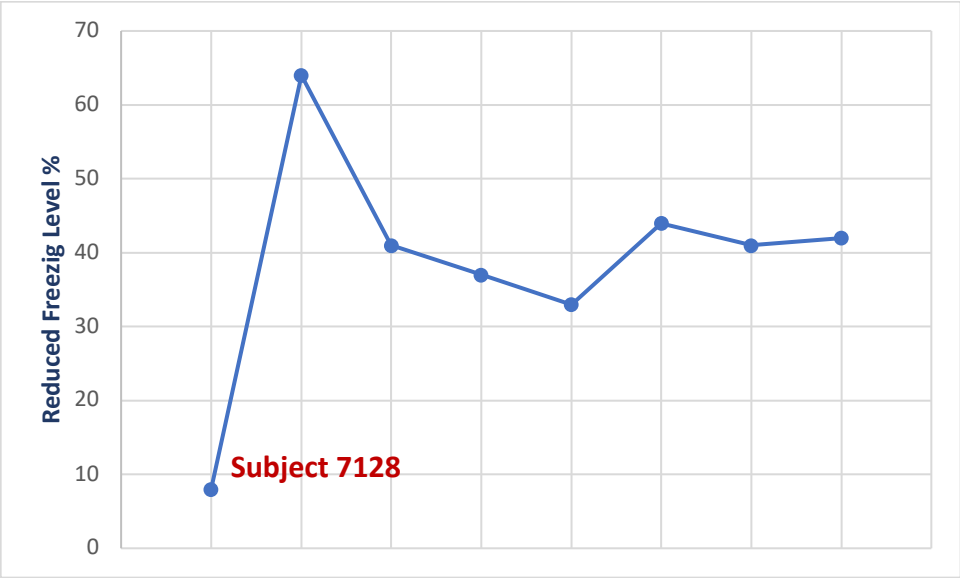


**Fig. 9 Lack of Reduced freezing level as evidence for the success of extinction learning in *7128*.** Reduced Freezing Level = 1- Freezing level in last extinction trial /Peak Freezing Level in FC. Individual dots represent reduced freezing level for the 6 mice included in previous analysis. The reduce Freezing level is 8 percent for subject *7128*, which is well below that of other mice, indicating poor extinction learning in *7128*.

**Freezing levels changes in mice with sufficient extinction learning**

To observe the possible effect of 40hz stimulation we predicted, the mice must have successfully acquired the extinction memory. While on average the 6 mice exhibited good extinction learning as previously shown, and all mice had a freezing level less or equal 50% by the last extinction trial, it was possible the poor extinction learning of one or more could be masked by the overall strong extinction learning in the group. To determine the success of extinction learning in each of the 6 mice previous analyzed, freezing level reduction rate was calculated by comparing the peak level of freezing during fear condition trials (from either the last 70s of FC2 or FC3) to that during the first 600s of the last extinction trial (Fig 9). Of the 6 mice, all had a freezing level reduction of more than 33% except for one (*7128*) where there was only 8% reduction, indicating

poor extinction learning. After excluding the mouse (*7128*), 2 way repeated measured ANOVA test was completed again for n=5 mice with full sets of freezing level data and good extinction learning. (Fig 10, Table 3,4) The overall results were similar to those before the *7218* exclusion with only significant main effect being trial type and no interaction effect. Though, the *p*-value was reduced from 0.24 to 0.18, $F(6.24) = 1.65$. The same post-hoc tests were performed, and similar results were obtained. (Fig 10, Table 3,4)

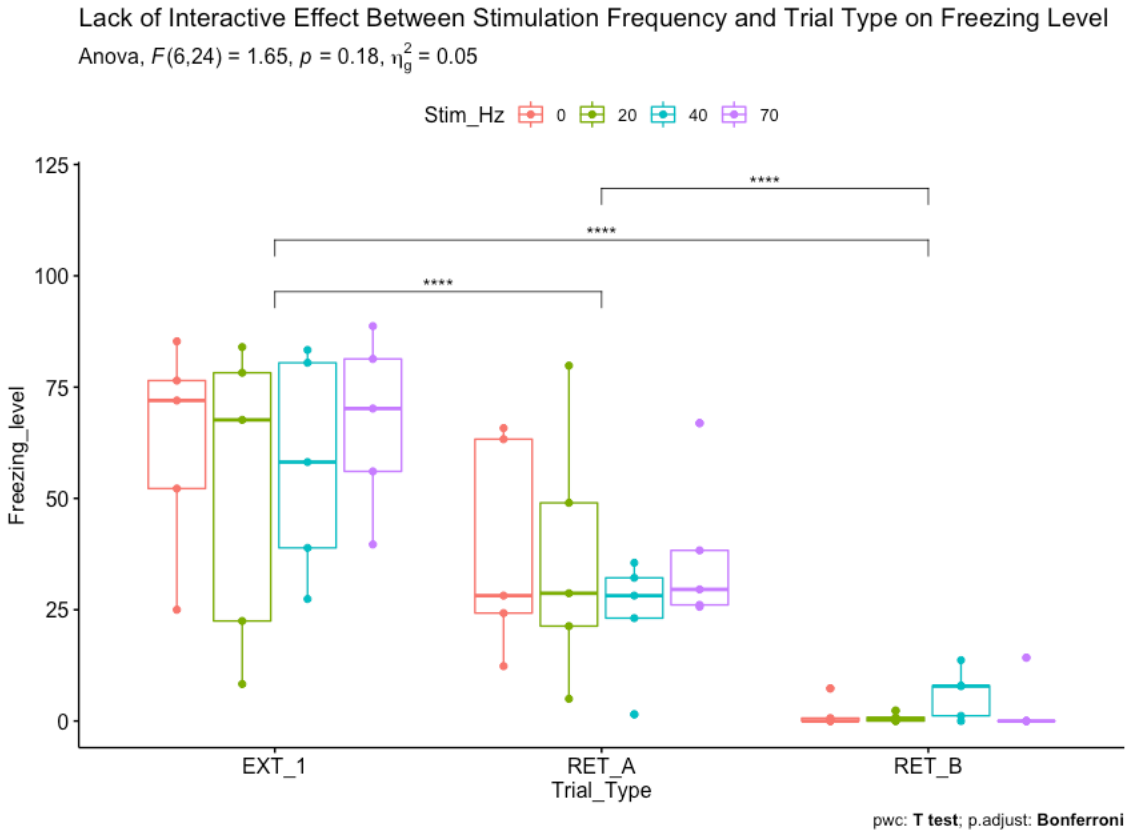


**Fig. 10 Lack of interactive Effect Between Stimulation Conditions and Trial Type on Freezing Level for Mice n=5**

| Effect | DFn | DFd | F | p | p<.05 | ges |
|---|---|---|---|---|---|---|
| Stim_Hz | 3 | 12 | 1.786 | 0.203 | | 0.024 |
| Trial_Type | 2 | 8 | 18.717 | 0.000961 | * | 0.632 |
| Stim_Hz:Trial_Type | 6 | 24 | 1.65 | 0.177 | | 0.047 |

**Table 3. 3 x 4 Repeated Measures ANOVA Statsitics for effects on the freezing level in mice n=5**

| / | group1 | group2 | n1 | n2 | statistic | df | p | p.adj | p.adj.signif |
|---|---|---|---|---|---|---|---|---|---|
| Freezing_level | EXT_1 | RET_A | 20 | 20 | 4.936706 | 19 | 9.16E-05 | 0.000275 | *** |
| Freezing_level | EXT_1 | RET_B | 20 | 20 | 10.28257 | 19 | 3.35E-09 | 1E-08 | **** |
| Freezing_level | RET_A | RET_B | 20 | 20 | 6.570092 | 19 | 2.73E-06 | 8.19E-06 | **** |

**Table 4. Pair-wise comparison T-test for significant Trail Type main effect on freezing level for mice n=5**

## Discussion

Previous literature suggests that extinction learning, the animal model for exposure therapy, introduces a competing extinction memory in addition to the original fear memory. Thus, the success and degree of extinction learning and exposure therapy might depend on whether retrieval memory is preferentially retrieved over fear memory. Clinically, understanding the oscillatory mechanisms mediating this competition of memory retrieval is important for developing new treatments, such as those using Transcranial Magnetic Stimulation, to prevent relapses of PTSD and increase the efficacy of exposure therapy.

From past studies and unpublished data in the Reijmers lab, we reasoned that 40Hz oscillation is likely to contribute to the establishment of extinction memory engrams and reactivation of these engrams during retrieval. We proposed a model where 40hz oscillation mediated by PV interneurons served as the mechanism that biases the retrieval competition for extinction memory. To verify the causal role of 40hz oscillations in promoting the extinction memory retrieval, we exogenously induced 40Hz oscillation at the BLA by controlling the activities of PV-interneurons using optogenetics. We also compared the effect of 40Hz with other stimulation frequencies (20Hz and 70 Hz), predicting that 40Hz should uniquely reduce the freezing level of mice during the retrieval session after fear and extinction learning.

Possibly due to the lack of subjects, I did not have the statistical power to verify or reject our predictions. Among mice with sufficient extinction learning, the freezing level only changed significantly across Ext_1, RET_A, and RET_B trials but not across different stimulation conditions. There was also no interaction effect between stimulation condition and trial type to warrant a further look into the effect of stimulation conditions on the freezing level in each trial type. It is possible that the effect size 40Hz stimulation is small and requires many more subjects to yield a significant result, but other problems may also complicate the picture. One possible complication might arise from the bilateral nature of virus injection and cannulae implantation. While optogenetic manipulation of brain regions bilaterally could amplify the effect on behavior, the trauma from implants and surgery induced to the brain may also result in more damage to the brain.

The greatest complications, however, arose from the nature of the optogenetic stimulation. Sustained gamma oscillation for 20 seconds is rare in BLA, where gamma oscillation tends to have a bursting nature (Bocchio et al., 2017, Stujenske et al., 2014). The sustained stimulation used in this experiment was powerful but also did not resemble endogenous gamma activities. The phase-amplitude coupling of gamma and other slower frequencies during extinction learning and retrieval of extinction memory also warrants a closer look. Increases in phase-amplitude coupling at BLA between theta and gamma between BLA gamma to theta frequency in other brain regions have been identified in fear memory learning (Bocchio et al., 2017). A similar mechanism may contribute to extinction memory learning, and thus the use of non-sustained, nested gamma (i.e. theta nested gamma) stimulation may more closely resemble endogenous activities. Furthermore, a closed-loop control manipulation of gamma activity may also be helpful despite difficulties in implementation (Kanta et al., 2019).

Current optogenetic stimulation in the study was applied without knowing the co-occurring oscillation activities in the BLA. The stimulation could interrupt already occurring oscillation, forcing the oscillation to shift its phase, which might subsequently interrupt already synchronized oscillatory activities. With a closed-loop system where the BLA local field potentials are being measured in real-time, we would have the option to amplify 40Hz oscillation upon its detection or erase endogenous 40hz oscillation by activating light-sensitive chlorine channels and observe the behavioral effects. We could induce 40hz oscillation when a mouse is freezing and observe if the freezing behavior is more likely to cease after the onset of stimulation.

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