SPECIFIC AIMS

The goal of this experiment is to explore the therapeutic effects of mindfulness on drug addiction, specifically nicotine. Mindfulness is a cognitive tool that focuses on self-awareness, compassion, and deference of judgement. Previous studies indicate individuals who practice long-term mindfulness show activation of different brain regions, suggesting neurophysiological changes related to practice. A previous study applied mindfulness to smoking cessation; though researchers found no significant effects in the short-term, the results showed significant changes for a longer duration of mindfulness practice. This result suggests neurophysiological changes of Mindfulness Training (MT) take time to progress—brain plasticity will adjust to drugs and lack thereof. Among these changes are effects to the dopamine (DA) reward circuitry, which addictive drugs target. We aim to find neurophysiological evidence of mindfulness in dopaminergic pathways of smokers, with the hopes that our findings will extend mindfulness as effective drug relapse prevention and help those with nicotine addiction. We will recruit volunteers who hope to quit smoking. Participants will be split into two groups, given either the current, standardized treatment program, or the standard with the addiction of MT.

Aim 1: **To Determine the Effects of MT on Withdrawal Symptoms**

During the first week of treatment for both programs (the first week of abstinence), participants will record their withdrawal symptoms in a journal once every hour they are awake. This includes a checklist of common withdrawal symptoms: headache, anxiety, nausea, and sweating (not due to physical activity), which participants will be asked to rate on a scale of 0 to 10 depending on the presence and severity of each symptom. We hypothesize that individuals who receive MT will report less withdrawal symptoms.

Aim 2: **To Determine the Effects of MT on Self-Reported Measures of Affect and Craving.**

Throughout the course of the 8-week treatment programs, each individual will self-report measures of craving and affect. Participants will self-report affect using the Positive and Negative Affect Schedule (PANAS) adapted for substance use, and self-report craving on a Likert scale from 1 to 7 as well as give brief descriptions of their experiences. Sessions will compare the levels of affect and craving between standard treatment and the addition of MT. We hypothesize that individuals undergoing MT will report less craving and negative affect.

Aim 3: **To Determine the Effects of MT on Cue-induced Dopamine Response**

We will administer brain positron emission tomography (PET) image scanning to all participants after the first week, and then again after the eighth week. Abused drugs are well known to affect the mesocorticolimbic dopamine reward and glutamatergic pathways of the brain. During each session, participants will be exposed to smoking cues (a cigarette, the smell of smoke) in the lab. The PET scan will image the brain using a tracer, looking for DA transporter activity. We hypothesize scans will reveal a difference in DA activity after eight weeks between individuals who completed MT and individuals who did not.

SIGNIFICANCE

This study explores MT as a new tool for rehabilitation and drug relapse prevention. Research has shown that mindfulness and meditation (practicing a state of mind) has neurophysiological effects; previously, researchers mapped out brain activity in inexperienced and experienced mediators and found that those more experienced had different brain activity, which resulted in decreased mind-wandering and more goal-directed task orientation (12). Since these findings have shown that consciously directing one's mind has physiological effects, there has been an interest in incorporating MT techniques to rehabilitation and relapse prevention, as current measures of effective treatment standards are combinations of medical and social programs.

In smoking cessation, a few pilot programs have already looked at the results of MT on craving and withdrawal symptoms, showing it as a therapy option to disassociate craving and smoking, including temporarily decreasing craving and withdrawal symptoms (2-3). Though mindfulness techniques may not reduce initial smoking urges after a temporary time, it may instead change responses to urges, giving it a place in rehabilitation therapy (4). In randomized clinical trials, individuals who went through MT had greater cigarette use reduction and maintained better results in 4 month follow-ups than current treatment standard programs by the American Lung Association (1).

Outside nicotine use, MT has also been researched in depressive symptoms, other substance use, and PTSD. MT was shown to weaken relationships between depressive symptoms and substance craving post-intervention, suggesting it is a better relapse treatment option that current standards (5). Between mindful and suppressive coping strategies in individual afflicted with PTSD and substance cravings, previous research has shown that the former had more positive, healthier results than the latter (6). Research implies mindfulness is a helpful tool to gain self-reflection and criticism, which are important to recovery (7).

Although a review of current literature shows promising directions, there is no standardized approach for MT as practical application for drug rehabilitation and relapse prevention. Mindfulness—the mind-body connection—seems to have practical application in the area of drug addiction. In this project we will look at the effects of mindfulness on smoking cessation compared to current treatment standards. We hope to improve clinical standards for treatment and add scientific, neurophysiological evidence for MT.

The positive impact of this research may extend to enhanced therapeutic practices for those struggling with substance addiction. With MT, former addicts can increase their sense of autonomy and change the way they perceive contextual cues. By improving standards of treatments and emphasizing internal change, we hope to prevent substance abuse relapse. Within the medical practice of substance treatment, MT could be an effective tool, decreasing the amount of individuals who relapse. Ultimately, we hope to add scientific background to a process that has more often than not been dismissed as alternative medicine in the past. This we

hope will bring a new perspective to drug treatment and improve the quality of life for many struggling addicts.

INNOVATION

This experiment will extend the understanding of MT—both affectively and neurophysiologically—in its effects on drug rehabilitation and relapse prevention.

1. MT, which incorporates compassionate, non-judgmental stance of self-awareness, is currently a fringe understanding in the field of addiction. Western medicine has only recently begun to explore the mind-body connection (affects and thoughts physically changing the body, and vice versa) in a picture of holistic body health. By detecting neural changes brought on through MT and focusing on scientific procedures among anecdotal evidence, we hope to shed light on a subject that has been stigmatized in the past as "alternative medicine", and better understand the clinical implications of MT.

2. Current literature reveals pilot MT programs for individuals who abuse alcohol and nicotine (1,7). While using similar measures and procedures (Likert-scale self-assessments, randomized, double-blind trails grouped into current treatment standards and those with the addendum of MT), we provide a more comprehensive framework including quantitative and qualitative self-reported measures of craving, withdrawal, and affect.

3. Participants will undergo PET imaging techniques to scan for DA activity in the brain. Past experiments have not explored the neurophysiological effects of mindfulness, especially in its application to drug rehabilitation and relapse prevention. We will analyze cue-induced DA response using the temporal resolution of the PET procedure. We hope to establish MT standards as evidenced by the short and long term effects on the DA reward system.

4. Previous experiments have relied on brief audio recordings for training (3,8); we will utilize a which offers MT sessions by an experienced instructor in group sessions, modified from previously established MT manual texts for drug relapse prevention (4,2). For our purposes, MT sessions will be tailored specifically to nicotine addiction.

APPROACH

Overall Design: This experiment will incorporate adult volunteers who struggle with nicotine addiction, split up into 1 of 2 groups: current treatment standards and standards with the addendum of MT. Participants will periodically self-report measures of withdrawal symptoms, affect, and craving. Within lab testing, they will go through PET imaging to record smoking cue-induced dopamine activity within brain regions.

Subjects: Participants will be recruited through localized media advertisements and flyers. Eligible participants 18 years or older who have unsuccessfully quit or have never attempted to

quit tobacco will be offered rehabilitative behavioral training. These participants are only eligible if they are not currently on psychoactive medications and not have met DSM criteria for other substance dependence in the past year. We aim to have at least 10 participants in each category between standard and MT addendum programs (a total of 20 participants).

Mindfulness Training Procedure: Volunteers will be split in half according to random assignment—individuals will undergo either the American Lung Association's FFS program (control group), or the program with the addition of MT (experimental group) (1). The experiment will consist of eight weeks of treatment for both groups—each individual will be asked to self-report measures during the course of the programs. Four mandatory sessions will be offered weekly.

Self-reported Measures of Withdrawal, Affect and Craving: During the first week of treatment, participants will be given a journal to record withdrawal symptoms they may have every 2 hours they are awake. Symptoms include headache, anxiety, nausea, and sweating (9). Each will be presented in checklist form with a scale of 0 to 10. Participants will be asked to rate the presence and severity of each symptom (0 being no symptom, 10 being very severe). In addition, participants will self-report measures of affect using PANAS adapted for substance use (10). Participants will also self-report craving on a Likert scale from 1 to 7. Affect and craving measures will be recorded five times a week throughout the 8-week program while withdrawal measures will be recorded once every two waking hours during the first week.

PET Imaging Procedure: Individuals are assessed for dopamine activity within the brain to look for active dopamine sites during 2-hour lab sessions of PET imaging, once a week after the beginning of the treatment program, and once after the eighth week, when the program is finished. An hour prior to the scan, each participant will be given an intravenous catheter at the arm with a solution containing tracers targeting the dA transporter ligand d-threo-methylphenidate. During the PET scan, each individual will be presented smoking cues. They will be in a room with the smell of smoke, and have a cigarette placed in front of them. After the cues are presented, PET images will be recorded using a CTI 931 scanner (Siemens, Knoxville, Tenn.) and rendered images will be compared (11, 14).

<u>Aim 1: Effects of MT on Withdrawal Symptoms</u>

Past research has shown mental calming exercises decrease anxiety and tension, with emphasis on mind-body connection (12). Self-reported withdrawal symptoms will only be recorded during the first week of treatment, once every two waking hours. By looking at this trend over time, we can analyze difference in presence and severity of withdrawal symptoms between those undergoing standard treatment and those undergoing treatment with the addition of MT. We predict that withdrawal symptoms will decrease over the initial week of drug abstinence across individuals who undergo standard treatment and individuals who have MT. However, we also predict that the latter will show significantly less withdrawal symptoms than the former for each day.

Aim 2: To Determine the Effects of MT on Self-Reported Measures of Affect and Craving.
MT focuses on maintaining attention of an individual's experiences and an attitude of acceptance toward the experience, ultimately recording an individual's own reflection process (13). Adapted from a previous manual that applies MT, we will have an experienced teacher lead four sessions every week for eight weeks. Participants will report affect using PANAS, a commonly used and well-established positive and negative affect scale, with an additional positive-negative Likert scale 7-point assessment (10). Participants will also self-report craving on a Likert scale (common self-report scale that is easily quantifiable) from 1 to 7, as well as give brief descriptions of their experiences. These measures will be recorded throughout the 8-weeks of treatment. Qualitatively we will look for patterns and word choice within the descriptions. Quantitatively, we will apply regression analyses to Likert-scale based questions. Our first session (initial week of abstinence) will compare the levels of affect between standard treatment and the addition of MT. These measures will be taken repeatedly across time, every two waking hours for first week followed by five times a week for the next seven. We predict that individuals who undergo MT will report less negative affect and craving than those who just undergo standard treatment.

Aim 3: Effects of MT on Regional Dopamine Activity in the Brain
We aim to systematically study the neurophysiological effects of MT by concentrating on dopamine activity within sub-regions of the brain. Current literature does not explore the technique at a neuronal level. We will administer PET image scanning after treatment at one week and eight week intervals. PET imaging will proceed in a double-blind study, where participants are unaware of the standard/mindfulness group differences, and the researchers administering PET image scans will be unaware of what group the individual is assigned to. The PET scan will image tracers that specifically target dopamine synthesis (11). The final 3D images will be processed through software and compared between those who received MT and those who did not. We hypothesize that PET scans will not reveal difference in dopamine activity and dopamine receptor numbers for the first week, as previous research shows no significant short-term effects (1). However, we also hypothesize that the scans will reveal a difference during eighth week between individuals who completed MT and those who did not.

Potential Pitfalls and Alternative Directions: A number of factors may come into play based on the methods and design of this study.
-Previous pilot applications of MT have focused on craving and cessation in a relatively short time frame (1, 2, 4). The length of time in this experiment (8 weeks) may not be long enough to see lasting effects of MT. Longer time lengths can help us more accurately measure the lasting effects of MT and relapse rates. In the future, we may focus on the effects of MT on cessation and relapse separately, recruiting people who have successfully quit but struggle with escalating craving.

-This study only looks at nicotine addiction. For future direction, MT can be explored in the context of other abused drugs such as cocaine and methamphetamine. MT may turn out to be more successful at treating one drug than another—further exploration would be required.
-Self-reported affect and craving measures may be skewed as individuals might feel the need to alter their answers to look more "successful" than they are. We will strive to preserve anonymity and a familial level of comfort for the participants. This is always the risk with self-reported measures.
-Participants themselves may not have completed the full program—considerations need to be made for incomplete trials, and whether or not their data should effect results.
-For PET imaging, our contextual cues may be too simple to replicate a realistic experience. Stressors are also not included in the experiments. Future incorporation and closer focus on these elements may increase validity pertaining to the experience of addition in reality.
-More refinement for PET imaging procedures could reveal a nuanced view into the DA reward pathway. This includes tracer for the synthesis and transportation of DA, specificity of D1 an D2 receptors, as well as consideration for Glutamatergic and GABAergic connections, and even brain region specificity such as the nucleus accumbens, ventral tegmental area, and the prefrontal cortex.
-The pool of participants itself may skew results—individuals are volunteers who sought help and have been motivated to action. Mindfulness training could be more or less effective in a more unbiased population, arguably for groups of people who need help the most but cannot seek it for whatever reason.
-We also did not include individuals who are currently on psychoactive medication and/or have met DSM criteria for other substance dependence. Abuse frequently follows comorbidity patterns with mental illness and simultaneous drug use. Current literature does not offer enough resources to understand how MT may play a part in treatment of comorbidities; we hope our research advances current field limitations.

References

1. Brewer, J. A., Mallik, S., Babuscio, T. A., Nich, C., Johnson, H. E., Deleone, C. M., ... & Carroll, K. M. (2011). Mindfulness training for smoking cessation: results from a randomized controlled trial. *Drug and alcohol dependence*, 119(1), 72-80.
2. Elwafi, H. M., Witkiewitz, K., Mallik, S., Thornhill IV, T. A., & Brewer, J. A. (2013). Mindfulness training for smoking cessation: Moderation of the relationship between craving and cigarette use. *Drug and alcohol dependence*, 130(1), 222-229.
3. Cropley, M., Ussher, M., & Charitou, E. (2007). Acute effects of a guided relaxation routine (body scan) on tobacco withdrawal symptoms and cravings in abstinent smokers. *Addiction*, 102(6), 989-993.
4. Bowen, S., & Marlatt, A. (2009). Surfing the urge: brief mindfulness-based intervention for college student smokers. *Psychology of Addictive Behaviors*, 23(4), 666.
5. Witkiewitz, K., & Bowen, S. (2010). Depression, craving, and substance use following a randomized trial of mindfulness-based relapse prevention. *Journal of consulting and clinical psychology*, 78(3), 362.
6. Garland, E. L., & Roberts-Lewis, A. (2013). Differential roles of thought suppression and dispositional mindfulness in posttraumatic stress symptoms and craving. *Addictive behavior*s, 38(2), 1555-1562.
7. Garland, E. L., Roberts-Lewis, A., Kelley, K., Tronnier, C., & Hanley, A. (2014). Cognitive and affective mechanisms linking trait mindfulness to craving among individuals in addiction recovery. *Substance use & misuse*,49(5), 525-535.
8. Ussher, M., Cropley, M., Playle, S., Mohidin, R., & West, R. (2009). Effect of isometric exercise and body scanning on cigarette cravings and withdrawal symptoms. *Addiction,* 104(7), 1251-1257.
9. Shiffman, S. M., & Jarvik, M. E. (1976). Smoking withdrawal symptoms in two weeks of abstinence. *Psychopharmacology*, 50(1), 35-39.
10. Thompson, E. R. (2007). Development and validation of an internationally reliable short-form of the positive and negative affect schedule (PANAS). *Journal of Cross-Cultural Psychology* 38 (2): 227–242. doi:10.1177/00220221062973
11. Elsinga, P. H., Hatano, K., & Ishiwata, K. (2006). PET tracers for imaging of the dopaminergic system. *Current medicinal chemistry*, *13*(18), 2139-2153.
12. Brewer, J. A., Worhunsky, P. D., Gray, J. R., Tang, Y. Y., Weber, J., & Kober, H. (2011). Meditation experience is associated with differences in default mode network activity and connectivity. *Proceedings of the National Academy of Sciences*, 108(50), 20254-20259.
13. Bishop, S.R., Lau, M., Shapiro, S., Carlson, L., Anderson, N.D., Carmody, J., Segal, Z.V., Abbey, S., Speca, M., Velting, D., Devins, G., 2004. Mindfulness: a proposed operational definition. *Clin. Psychol*. 11, 230–241.
14. Volkow, N. D., Chang, L., Wang, G. J., Fowler, J. S., Leonido-Yee, M., Franceschi, D., ... & Logan, J. (2001). Association of dopamine transporter reduction with psychomotor impairment in methamphetamine abusers. *American Journal of Psychiatry*.

Fear Memory Expression in Hippocampal Sub-regions: Zif268 Gene Expression

Introduction

In recent years, many resources have gone into research on memory retrieval and system consolidation. In current literature, Wiltgen and Tanaka (2013) defined system consolidation as an incremental process that occurs after learning, transferring information from short to long term memory. Once consolidated, memories that initially required the hippocampus (HPC) become independent of the structure, and are more generalized—however, research continually shows that recently formed context memories, for example fear memories, require HPC during retrieval (Wiltgen & Tanaka, 2013).

An experiment conducted by Zelikowsky, Bissiere, and Fanselow (2012) revealed that though explicit memories can be acquired while the hippocampus is incapacitated, they do not last long. By lesioning the dorsal HPC in rats and putting them through fear conditioning, Zelidowsky et al. found a decay in explicit fear expression across time, suggesting that the memory could not be consolidated from recent to remote without HPC, even when formed outside the structure. Within the HPC itself, Yoon and Otto (2007) previously found dorsal and ventral sub-regions may contribute differentially to the underlying processes in trace fear conditioning. Pre-training lesions in Dorsal HPC had no effect on acquisition of trace-conditioned fear, while post-training lesions in the same area impaired fear expression (Yoon & Otto, 2007).

The roles of hippocampal sub-regions in memory consolidation must still be explored. Pierson, Pullins, & Quinn (2015) ran trace fear conditioning in rats infused with either AMPA antagonist CNQX or vehicle control into either Cornu Ammonis 1 (CA1) or dentate gyrus (DG) sub-regions. Pierson et al. (2015) found rats infused with CNQX in CA1 and DG had lower contextual fear, indicating both contributed to expression of fear-conditioned memories. CNQX

infusion into DG revealed lower freezing rates, suggesting DG is more important than CA1 in expression of trace fear-conditioned memories. This result is supported by previous research conducted by Czerniawski, Yoon, and Otto (2009), which revealed inactivation of CA1 had no significant effect on trace fear conditioning while inactivation of DG did. Through selective lesions, Lee and Kesner (2004) found CA3, CA1, DG contributed to contextual memory formation, but during retrieval CA3 was not active in contextual fear after a long time period of time (24 hrs) while CA1 and DG were. These results indicated time-dependent differences in hippocampal subregion contribution to contextual fear memory consolidation and reconsolidation (Lee & Kesner, 2004).

Beyond behavioral analysis, underlying neuronal circuitry and gene expression have been explored for memory reconsolidation. Bozon, Davis, and Laroche (2003) found the immediate early gene (IEG) zif268 was required for reactivation/reconsolidation of long-term memory. Bozon et al. (2003) showed zif268 mutant mice were impaired in long-term memory retrieval tasks, indicating IEG-mediated activity is important for the storage of newly formed and reactivated recognition memories. Weitemier and Ryabinin (2004) found zif268 levels in DG upon fear memory retrieval was higher in trace-conditioned than delay-conditioned rats. Hall, Thomas, and Everitt (2001) found an increase in zif268 expression within CA1 during contextual fear retrieval.

Antoine, Serge, and Jocelyne (2014) looked at the neural circuitry in HPC involved with the consolidation and reconsolidation of contextual fear conditioning memories by tracking the MAPK/ERK1/2 pathway. Antoine et al. (2014) tracked immunoreactive P-ERK1/2-positive cells in CA1, CA3, and DG sub-regions, and found contextual fear conditioning memory retrieval was associated with activity in the DG. Results showed an increase of immunoreactive cells in CA1 did not correlate to freezing response (increase may have occurred through contextual exposure),

while an increase in CA3 did (increase only found in fear conditioned groups). Molecular events controlled by ERK1/2 were then explored, including IEG zif268 protein expression, which increased after contextual memory retrieval in the DG (Antoine et al., 2014).

As fear memory retrieval has been correlated with the transcription of IEG zif268, our study considers the gene expression in a comprehensive and previously unexplored direction. We aim to narrow down behavioral freezing and zif268 gene expression activity in the hippocampal subregions DG, CA1, and CA3 through trace, delayed, and unsignalled fear conditioning. We will look at context fear (environmental) and cue fear (tonal) retrieval. We hypothesize that zif268 gene expression will correlate with freeze response and that expression will be found in different hippocampal sub-regions depending on initial fear conditioning and retrieval type. We discuss patterns further by looking at support from past literature in the "Expected Results" section.

Method

57 adult male Long-Evans rats from Harlan, Indianapolis (IN), approximately 90 days of age, were used in these experiments. They were fed *ad libitum* throughout, and housed two to a cage in a temperature and humidity controlled room with a 12:12 hour light/dark cycle. All experiments occurred during the light portion of the cycle. Experiments were approved by the Miami University Institutional Animal Care and Use Committee in accordance with NIH experimental animal guidelines. Rats were handled for five consecutive days prior to the start of fear conditioning in an effort to acquaint them with lab technicians.

Experimental Design

Two main independent variables were of interest: conditioning procedure and test type. Conditioning procedure consisted of three groups: trace, delay, and unsignaled conditioning. Test type consisted of two groups: context (in original training context A), and tone (within context B). On day one, fear conditioning commenced in context A, a well-lit, vanilla-scented rectangular room; context B, by contrast, was a dark, vinegar-scented triangular room used in memory retrieval for tone testing and control groups (see Fig. 1). Rats were pre-exposed to context B on day two to overcome any novel effect of a new environment that might occur.

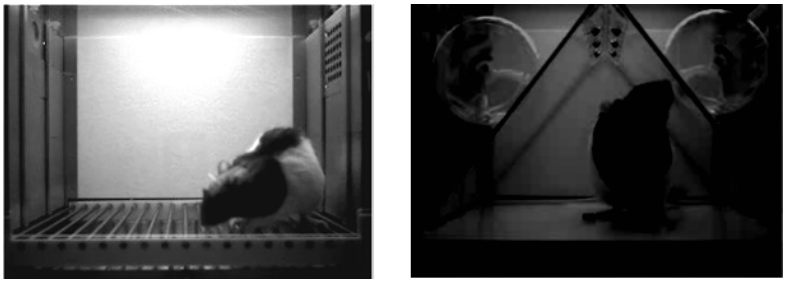

Figure 1. The different contexts A (left) and B (right) in comparison

Six groups of eight rats were placed in all possible conditioning procedure and test type combinations using a 3 (trace, delay, and unsignalled) x 2 (context and tone) experimental design, with an additional control group of nine rats. In the control group, a third went through trace, a third delay, and a third unsignalled fear conditioning (three rats per condition). The control group did not go through memory retrieval, and were instead placed in context B without tone.

Procedure

On day one, all rats were placed in Context A for fear conditioning. Rats undergoing trace fear conditioning had ten trials; to keep roughly the same associative strength, rats in delayed and unsignalled fear conditioning had three trial. Each trial for the fear conditioning lasted 256 seconds. A three minute baseline where no stimulus was given occurred prior to the first trial of trace and delayed fear conditioning. In trace conditioning, 256 second trials occurred where a tone (conditioned stimulus, 2KHz, 75dB) lasted 16 seconds, followed by a trace interval of 28 seconds and then a two second foot shock (unconditioned stimulus, 0.9mA) immediately afterward. In delayed conditioning, the same trial duration, shock, and tone were used. However, no interval between tone and shock was created—in the first 16 seconds of a trial, where the tone played, the last two seconds overlapped simultaneously with foot shock. In unsignalled conditioning, there was shock at trace intervals but no tone pair (context conditioning was focused on).

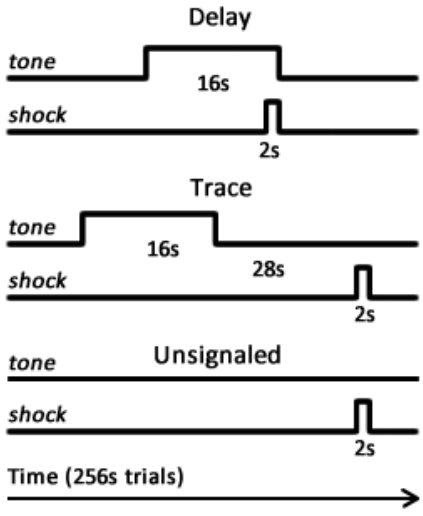

Figure 2. Tone-shock training pairs

On day two, all rats were placed into context B for pre-exposure to the environment, where they could freely roam for 45 minutes. On day three, researchers conducted fear-testing memory retrieval. In the context test, rats were placed in context A for eight minutes and measured for freeze response. In the tone test type, rats were placed in context B, given the tone three times for sixteen minutes and measured for freeze response. Freezing was monitored by a progressive scan video camera with a light filter (VID-CAM-MONO-2A, MED-Associates)

connected to a computer running Video-Freeze software (MED-Associates). In the control group, rats were placed in context B for sixteen minutes without tone.

Immunohistochemistry

Researchers waited for the peak of Zif268 gene expression (roughly 30 minutes) from the start of fear testing before injecting them with euthasol as an anesthetic 5-10 minutes prior to the 30-minute mark (to allow time for for anesthetic to take effect). Rats were given an intercardial profusion with cold 200ml phosphate-buffered saline solution to flush out blood, and a 400ml 4% paraformaldehyde solution to fix the brain tissue. The brain samples were stored in a 20% glycerol and phosphate buffer for at least 72 hours at 4 degrees. To determine brain slices, researchers took three separate points anteroposterior (AP) to bregma: -2.64 mm, -3.48 mm, and -4.36 mm (see Fig. 3). Brains were sliced into 40 μm coronal sections using a microtome and placed in the phosphate-buffered saline solution with 0.1% Sodium Azide, stored at 4 degrees.

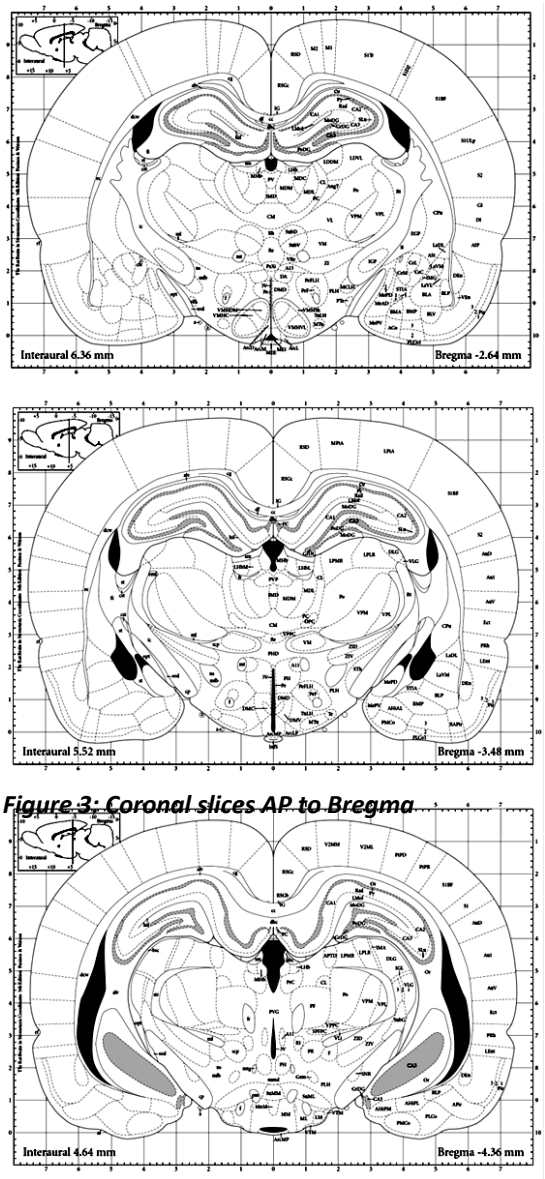

Figure 3: Coronal slices AP to Bregma

Brain samples were placed into well plates with 10 ml of solution in each well. First, tissue was placed in a .1% Triton-X + 1% BSA in TBS solution for an hour, a blocking buffer that prevented background staining. Samples were then placed in a solution containing specific primary antibodies derived from rabbits, EGR-1 15F7 Rabbit mAb (1:1000) overnight at room temperature, which caused antibodies to to Zif268

proteins On the second day, samples were placed into a secondary antibody derived from goats, Anti-rabbit in TBS + BSA (1% BSA, 10ml TBS) solution at room temperature for one hour, which bound to primary antibodies. Between washes, Tris-buffered Saline (TBS) was used to clear off excess primary and secondary antibodies. Samples went through three washes of TBS at 10 minutes each wash. Avidin-Biotin Complex (ABC) was then used to bind the biotin-laden secondary antibodies at room temperature for one hour, causing signal amplification. The samples were washed again with TBS, at 10ml for 10 minutes three times. A final 3,3'-Diaminobenzidine (DAB) solution was made with buffer, DAB, and H_2O_2. Slices were transferred to the DAB solution, then washed again with TBS at 10ml for 10 minutes three times.

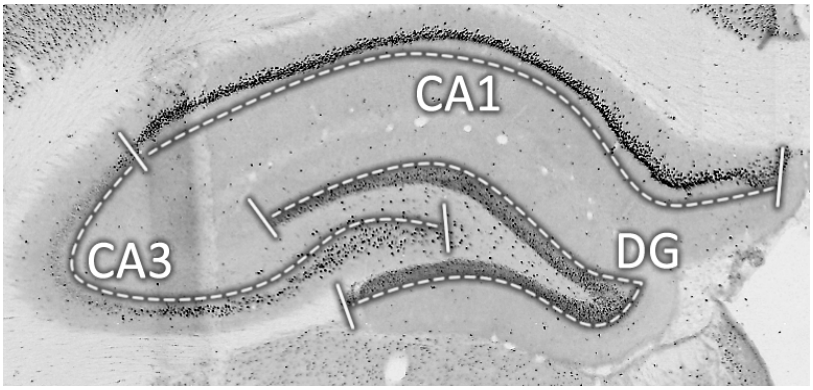

Figure 4: Dashed lines marking sub-regions, stained for Zif268 proteins

The DAB oxidation reaction created brown precipitate, the cell body staining used to visualize Zif268 protein expression (see Fig. 4).

On day three the ethanol precipitation method was used, where slides were dipped once in TBS, then rinsed for three minutes each using 70%, 80%, and 95% EtOH solutions consecutively, with two finals rinses of 100% EtOH and dip in xylene substitute (3 minute each).

Using image analyzing software, microscopy images were post-processed through adjustments such as change of threshold and contrast to better show brown precipitate. Precipitate coordinates were calculated by the software, and the average number of stained cells within the hippocampal sub-regions CA1, CA3, and DG in respect to the different types of fear

conditioning and fear testing were analyzed. A one-way ANOVA analysis with the three test groups (unsignalled, trace, and delay) was conducted.

Expected Results

For our behavioral predictions, we expect the context and tone fear-conditioned rats to freeze in the trends indicated by the graphs in Figure 5 below.

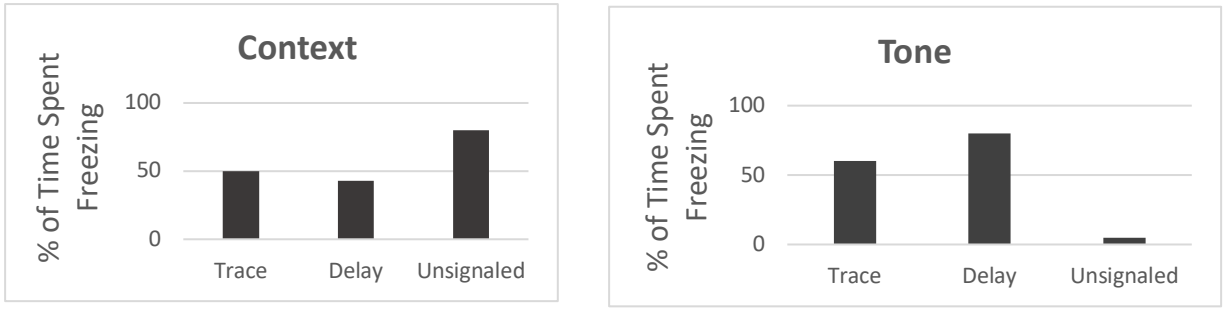

Figure 5: Graphs depicting freezing behavior based on training type

We predict a significant increase in freezing of trace-conditioned rats under contextual-retrieval (Pierson et al., 2015). Trace interval creates competing associative strengths between the environment and the cue, and both are valid in triggering fear; in Pavlovian conditioning, associative competitions selectively strengthens a dominant circuit over a secondary circuit (Fanselow, 2010). Thus, we also predict a significant increase in freezing of this group under tone retrieval. In contextual retrieval, we predict significant freezing in delay-conditioning that is less than trace because the trace interval holds less association. By contrast, we predict delay-conditioned rats will freeze more than trace-conditioned in tone retrieval because the tone-shock pair has strong associative strength (tone stronger than context). Finally, we believe the largest percentage of time spent freezing in the context graph will be unsignalled-conditioned rats, because they have no competing associative strengths of tone to distract from environment. Conversely, we predict the lowest percentage of time spent freezing in the tone graph to be in this group because they have no reason to fear the tone, as it was never paired with foot shock.

For zif268 gene expression, we predict the number of cells shown in Figure 6 below:

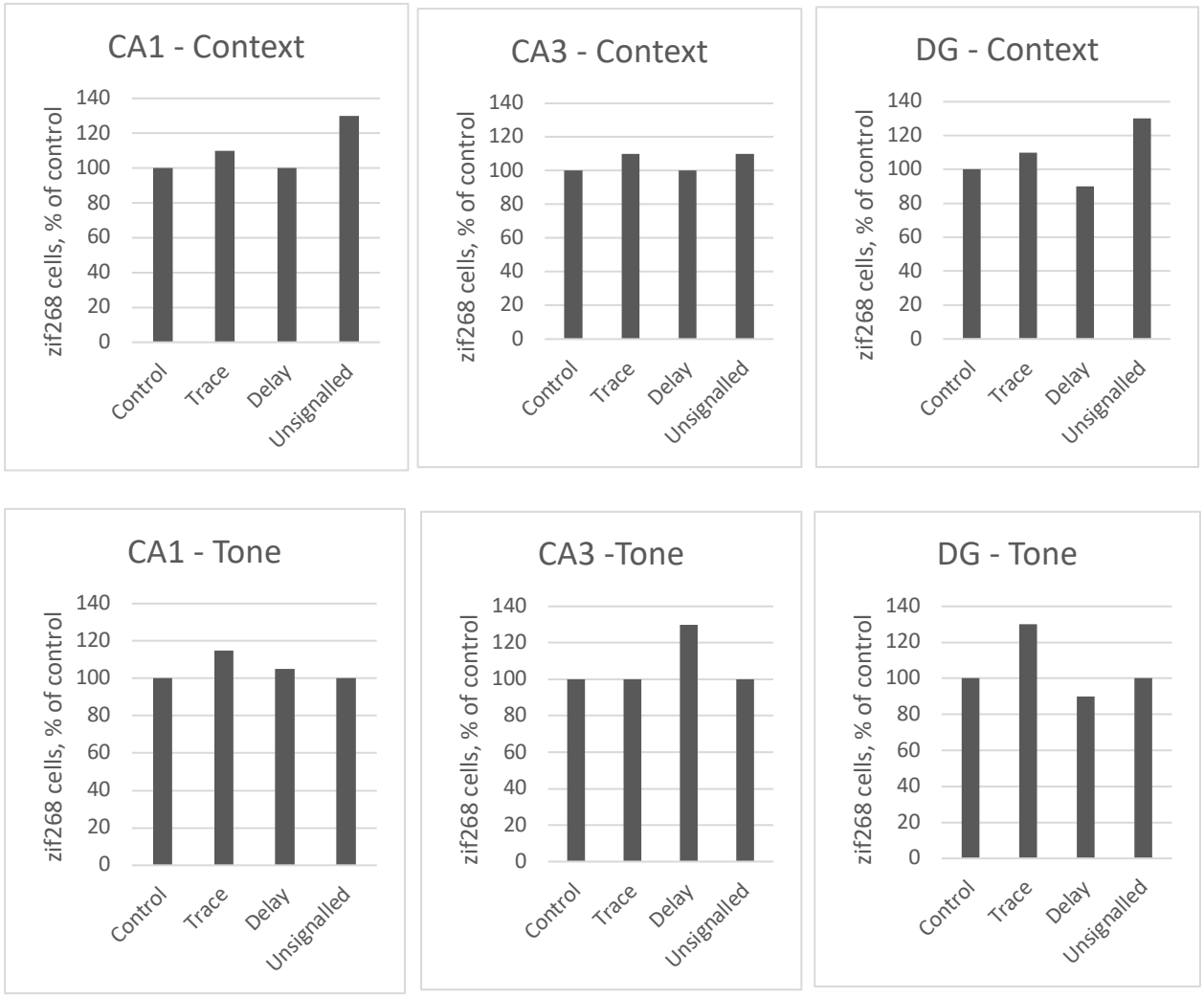

Figure 6: Graphs depicting # of Zif268 proteins found in respective conditions

Through previous research conducted by Pierson et al. (2015), we predict CA1 contributes to contextual fear and slightly to trace fear conditioned memory expression. Hall et al. (2001) also supported high zif268 in CA1 for context. Thus, the number of zif268 cells would be highest in the unsignalled-context, and only slightly higher than control for trace-context. Delay would remain around control because it was strongly paired with tone, which was not tested for. For CA1 under tone, we believe trace will have a bigger zif268 expression because of its associate strength with tone, with a slight delay effect and no unsignalled effect.

In CA3, Antoine et al. (2014) found P-ERK1/2+ cells increased in fear-conditioned groups, which correlated with freezing response. As the pathway leads to zif268 gene expression, we predict number of zif268 cells to correlate to freezing response previously predicted in Figure 5. Lee and Kesner (2004) reported results that CA3 did not contribute to contextual fear, thus delay is the same as control in context and highest in tone.

For DG, Lee and Kesner (2004), Antoine et al. (2014), and Pierson et al. (2015) all showed experimental results that the sub-region contributed to contextual fear. Pierson et al. (2015) and Czerniawski et al. (2009) also showed that DG significantly contributed to trace-conditioned fear expression. Antoine et al. went on to show zif268 gene expression was high in contextual fear retrieval, and Weitemier and Ryabinin (2004) previously showed that zif268 gene expression was higher in trace than delay. Thus, we predict significant increase in the level of zif268 proteins in unsignalled-context and trace-tone.

This study looks at hippocampal sub-region expression of zif268 protein in the context of fear memory retrieval, but each microscopy image was only one snapshot in time. Mokin and Keifer (2005) conclude that conventional IEG imaging methods like the one used in this experiment limit behavioral comparisons to between-subject design. We can get a more comprehensive look at memory consolidation and reconsolidation roles of HPC sub-regions by looking at different points of time. As Jones et al. (2001) found, zif268 is essential for consolidation from short- to long-term memory, and synaptic plasticity for long-term memory expression. Research can explore zif268's at different points of memory creation and retrieval.

References

Antoine, B., Serge, L., & Jocelyne, C. (2014). Comparative dynamics of MAPK/ERK signalling components and immediate early genes in the hippocampus and amygdala following contextual fear conditioning and retrieval. *Brain Structure and Function*, *219*(1), 415-430002E

Bozon, B., Davis, S., & Laroche, S. (2003). A requirement for the immediate early gene zif268 in reconsolidation of recognition memory after retrieval. *Neuron*, 40(4), 695-701.

Czerniawski, J., Yoon, T., & Otto, T. (2009). Dissociating space and trace in dorsal and ventral hippocampus. *Hippocampus*, 19(1), 20-32.

Fanselow, M. S. (2010). From contextual fear to a dynamic view of memory systems. *Trends in cognitive sciences*, 14(1), 7-15.

Hall, J., Thomas, K. L., & Everitt, B. J. (2001). Cellular imaging of zif268 expression in the hippocampus and amygdala during contextual and cued fear memory retrieval: selective activation of hippocampal CA1 neurons during the recall of contextual memories. *The Journal of neuroscience*, 21(6), 2186-2193.

Jones, M. W., Errington, M. L., French, P. J., Fine, A., Bliss, T. V. P., Garel, S., ... & Davis, S. (2001). A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nature neuroscience,* 4(3), 289-296.

Lee, I., & Kesner, R. P. (2004). Differential contributions of dorsal hippocampal subregions to memory acquisition and retrieval in contextual fear-conditioning. *Hippocampus*, 14(3), 301-310.

Mokin, M., & Keifer, J. (2005). Expression of the immediate-early gene–encoded protein Egr-1 (zif268) during in vitro classical conditioning. *Learning & Memory*, 12(2), 144-149.

Pierson, J. L., Pullins, S. E., & Quinn, J. J. (2015). Dorsal hippocampus infusions of CNQX into the dentate gyrus disrupt expression of trace fear conditioning. *Hippocampus*.

Weitemier, A. Z., & Ryabinin, A. E. (2004). Subregion-specific differences in hippocampal activity between Delay and Trace fear conditioning: an immunohistochemical analysis. *Brain research*, 995(1), 55-65.

Wiltgen, B. J., & Tanaka, K. Z. (2013). Systems consolidation and the content of memory. *Neurobiology of learning and memory*, 106, 365-371.

Yoon, T., & Otto, T. (2007). Differential contributions of dorsal vs. ventral hippocampus to auditory trace fear conditioning. *Neurobiology of learning and memory*, 87(4), 464-475.

Zelikowsky, Moriel, Stephanie Bissiere, and Michael S. Fanselow. Contextual fear memories formed in the absence of the dorsal hippocampus decay across time. *The Journal of Neuroscience* 32.10 (2012): 3393-3397.