ABSTRACT

HARP, SAMUEL JESSE. Differences in Fentanyl-Induced Conditioned Place Preference and Open Field Activity in A/J and B6J Mice; Potential Role for Glyoxalase 1. (Under the direction of Dr. Emilie Rissman).

All behavior, including behavior classified as a disorder, is the product of genetic and environmental forces. Drug addiction is no exception, and ongoing research attempts to understand both of these influences in the context of addiction. Mice are useful tools for understanding the genetic basis for behavior, and here we investigate strain differences in A/J and C57BL/6J (B6J) mice for fentanyl (0.2 mg/kg)-induced behavior. These two strains were selected because they vary in their response to other drugs like nicotine and cocaine. We tested mice using an eight-day conditioned place preference (CPP) protocol and an open field (OF) task. We also examined whether fentanyl administration changes expression of Glyoxalase 1 (Glo1), a gene that has previously been implicated in anxiety-like behavior and ethanol self-administration. We hypothesized that B6J mice would show a greater CPP and locomotor response to fentanyl, consistent with previous literature on B6J mice and other opioids. B6J mice did exhibit larger responses to fentanyl in both the CPP task and the OF task, and both strains showed reduced Glo1 expression in the prefrontal cortex after fentanyl administration.

To determine whether manipulation of Glo1 would affect fentanyl-induced behavior, we used B6J mice in an additional open-field study using pBBG, a Glyoxalase 1 inhibitor. 12.5 mg/kg pBBG reduced fentanyl-induced locomotor activity in the open field, supporting the hypothesis that Glo1 is involved with fentanyl-induced behaviors in mice. We propose that Glyoxalase 1 should be studied further as a potential pharmacological target for addiction treatment.
Differences in Fentanyl-Induced Conditioned Place Preference and Open Field Activity in A/J and B6J Mice; Potential Role for Glyoxalase 1

by
Samuel Jesse Harp

A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Master of Science

Genetics

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APPROVED BY:

______________________________
Dr. Emilie Rissman
Chair of Advisory Committee

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Dr. Cynthia Kuhn

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Dr. John Godwin
DEDICATION

Dedicated to all of the frontline workers of the COVID-19 pandemic, especially healthcare workers, who have all taken on enormous risk and responsibility and have persevered in the face of enormous challenges.
BIOGRAPHY

I grew up in Canton, Georgia, and graduated from Cherokee High School in 2015. My family lived in a somewhat rural area, and this made for a great childhood- exploring in the woods with my brother, planting vegetables in our greenhouse, and shooting BB guns off the back porch. During high school, I developed a real interest in understanding behavior and the reasons that people do what they do. I attended college at the University of Georgia, and studied psychology and biology. While in college, I waited tables at a local country club, and worked for a funeral home doing “pickups” after hours. After graduation, I worked in a plant genetics lab for a short period, and gained experience that was more relevant to what I wanted to do as a career. Soon after, I came to the Genetics program at North Carolina State University. I’ve worked in Emilie Rissman’s lab for the last year and a half, and have learned an enormous amount about working with mice, designing experiments, and interpreting results. All of these will be useful to me throughout my career as a scientist. Although I was originally on the PhD track in the Genetics program, some major life changes have caused me to re-think what the best path for me is, and I am thrilled to now be submitting my work for the degree of Master of Science in Genetics.
ACKNOWLEDGEMENTS

Thank you first and foremost to my parents and to my brother Alex for being supportive of me long before I started graduate school, as well as during my time at NC State.

Thank you to Erin for also being incredibly supportive, and for being such an important part of my young adulthood.

Thank you to Emilie for being such a caring and helpful PI, and for helping me become a more knowledgeable scientist.

Thank you to other members of the Rissman Lab- former (Mary and Christina) and current (Hugh, Srikar, Ryn, Aaron) for being great resources and doing the necessary work to keep the lab going.

Thank you to the Lucas lab, the Lynch lab, and the Farber lab for teaching everybody something during joint lab meetings and collaborations on projects.

And of course, thank you to Clay Rouse, for helping us with surgeries.
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Introduction

Drug overdose deaths in the United States recently reached an all-time high of 81,230 for the 12-month period ending in May 2020. An increase in overdose deaths was evident in 2019 before the COVID-19 pandemic, and the trend has only accelerated since. Reports from state health departments indicate that fentanyl, a synthetic and highly potent opioid, is the primary driver of these trends in recent years (Centers for Disease Control and Prevention). Research on substance abuse has led to valuable discoveries of drugs that are used to assist with drug detox and with relapse prevention (Douaihy, Kelly, & Sullivan, 2013). Drug use disorders are the product of both genetic and environmental conditions, and are often caused by an interaction between the two (Kendler et al. 2012). Data from twin studies shows a significant role of Single Nucleotide Polymorphisms (SNPs) in the development of opioid dependence (Brick et al. 2019). There is some variability between studies (which may use different subjects or criteria), but a common estimate for the heritability ($h^2$) of opioid use disorder (OUD) is 0.40 - 0.50. The heritability of analgesic response to opioids is similar (Crist, Reiner, & Berrettini 2019; Gillespie, Bates et al., 2019; Angst et al., 2012; Brick et al. 2019). This correlation points to a possible connection between the overall physiological response to opioids (including factors like the effectiveness of opioids to relieve pain in an individual) and the susceptibility of an individual to developing an OUD. In fact, some of the same mutations in genes like mu opioid receptor one ($\text{OPRM1}$) have been associated with both heroin addiction and with clinical effectiveness of morphine (Lotsch & Geisslinger, 2005). This is a critical point to study further, as patients for whom opioids work most effectively may be more likely to develop an addiction.

Inbred mouse lines are a useful tool for answering questions about complex behaviors like drug addiction. Mouse strains show extensive variation in a number of physiological and behavioral traits, including responses to rewarding stimuli (Dickson et al. 2015). There are a number of genomic resources that are available to answer questions about the underlying
genetics of the behavior. The majority of preclinical data on drugs of abuse comes from studies using a small number of outbred rat strains (including Sprague Dowley and Long Evans) and one inbred mouse strain (C57BL/6J). These strains are selected for their high vulnerability to the reinforcing effects of drugs, especially cocaine. Inbred lines are useful for pinpointing genetic differences that may contribute to a variety of phenotypes, including drug-induced behaviors.

For example, intravenous (IV) self-administration (SA) studies comparing DBA/2J and C57BL/6J (B6J) mice have shown that B6J mice readily acquire morphine SA, while DBA/2J mice fail to acquire at all (Tapocik et al. 2013). After morphine exposure, hundreds of genes are differentially expressed in B6J ventral striatum, and only a tiny fraction of these genes are differentially expressed in the ventral striatum of DBA/2J (Tapocik et al. 2013).

Previous studies are informative about the differences between strains in drug-induced behavior with mice, and also show differences in the same strain’s response to drugs in different classes, such as ethanol and cocaine (Fish et al. 2009). Many classes of drugs have been used in research on addiction with rodents, and we suggest that fentanyl is under-studied compared to other drugs of abuse, given that synthetic opioids like fentanyl have caused more overdose deaths than any other class of drug during the last five years (National Institute on Drug Abuse, 2021). The focus of the current study is whether strain differences exist in response to fentanyl, and whether these differences are associated with expression of Glo1, a candidate gene shown by Barkley-Levenson et al. (2018) to influence ethanol consumption in mice. We show that A/J and C57BL/6J (B6J) mice differ in their locomotor response to fentanyl, and also differ in the formation of Conditioned Place Preference (CPP) after conditioning. Expression of Glo1 in the prefrontal cortex also differed between strains, and was modulated by fentanyl treatment. These differences in behavior and gene expression between strains are crucial to understand, because they often lead to the discovery of genes that influence drug-related behaviors, or even novel therapeutic targets for addiction treatment.
Using mice in tasks like the open-field task to identify associations between genes and behavior can often translate directly to relevance in humans. One example of this is Casein Kinase 1 Epsilon (CSNK1E), which was identified by Palmer et. al (2005) as being involved with locomotor response to methamphetamine. Expression differences for CSNK1E were identified between one mouse line bred for high methamphetamine (MA)-induced locomotor activity, and the other bred for low MA-induced activity. Veenstra-VanderWeele et. al (2006) later surveyed a population of 100 human volunteers given either a placebo, low MA dose, or high MA dose. One SNP in CSNK1E was identified that had significant effects on how strongly participants in the low dose group “felt the drug”, as well as the amount of euphoria experienced by the low dose group. Even with a relatively low sample size, the differences in perceived effects were robust between groups with different CSNK1E alleles. This study demonstrates the value of research that is translated from mice to humans. It also suggests that many of the same genes that control drug-induced locomotor activity may also influence euphoria or other elements associated with addiction.

The current study seeks to identify strain differences in behavioral response to fentanyl and genes that may facilitate vulnerability to either the rewarding or behavioral effects of fentanyl. In the first stage of the experiment, behavioral responses to fentanyl were compared in two inbred mouse strains; B6J and A/J. Then, differences in gene expression between A/J and B6J were examined for mice that received either fentanyl or saline injections. These two strains were selected because they show significant differences in a variety of relevant behaviors, including exploratory locomotor activity, voluntary ethanol consumption, and acquisition of cocaine self-administration (Thomsen & Caine 2011; Matthews et al. 2008; Brodkin et al. 1998). B6J mice have been shown to exhibit significantly higher locomotor activity in the open-field test, including after acute exposure to cocaine (Wiltshire et al. 2015). Additionally, A/J mice have been found to be less sensitive than B6J to fentanyl's depressive effects on respiration.
(Fechtner et al. 2016). Because males were exclusively used in many of these studies, the current study builds on previous work by examining outcomes for both males and females. Based on previous literature comparing the A/J and B6J strains, we hypothesized that B6J mice would display stronger CPP for fentanyl than A/J mice after conditioning, and that B6J would exhibit greater fentanyl-induced locomotor activity in the open field.

Chromosome substitution lines using A/J chromosomes in a B6J background showed that both fentanyl- and methamphetamine-induced spontaneous and conditioned activity varied between the two inbred strains, and that a quantitative trait locus (QTL) on Chromosome 11 was responsible. (Bryant, Chang, et al., 2009). In a panel of 39 BXD strains trained in IV-self-administration, a QTL on chromosome 11 was also found to shift the dose-response curve for cocaine. These studies demonstrate the value of using multiple strains to uncover QTL influencing addictive behaviors. Recombinant inbred lines can be especially useful for this purpose. A/J and B6J mice are two of the eight paternal mouse strains used to produce collaborative cross (CC) and diversity outbred (DO) mice, both of which are widely used in addiction research, and exhibit a range of behavioral responses to various drugs (Saul, Philip & Reinholdt, 2019). CC mouse lines have been assayed for spontaneous activity, conditioned place preference (CPP) and intravenous (IV) self-administration in response to cocaine, with different lines showing a wide range of drug-induced behaviors (Schoenrock et al., 2020). Lines created from recombination between B6J and other inbred lines (like DBA/2J) have been tested on several novelty tasks, and have also been used in cocaine self-administration studies (Dickson et al. 2016).

B6J mice are commonly used to study addictive behaviors in mice, presumably because they show greater responses to drugs of abuse than most other inbred strains (Crawley & Paylor, 1997). There have also been a number of previous studies which used the A/J mouse strain. Kutlu et. al (2015) used eight inbred mouse strains, including B6J and A/J, to assess
development of CPP after multiple conditioning sessions with nicotine. One side of an apparatus was paired with nicotine, and the other was paired with saline. After conditioning, three of the mouse strains (including B6J) showed nicotine-induced place preference, and four strains (including A/J) did not show place preference. Silvers et. al (2008) investigated the effects of a foot shock on consumption of a 10% ethanol solution by multiple mouse strains, including B6J and A/J. A mild foot shock increased the consumption of ethanol in B6J, but not for A/J mice. Spontaneous locomotor activity in response to a cocaine injection was robust in B6J but barely detectable in A/J mice (Thomsen & Caine, 2011, “False positive…”). Finally, in a self-administration study with cocaine, all of the B6J mice acquired self-administration, while only two out of seven A/J mice acquired. Reinstatement responses of the A/J mice were significantly lower than for B6J (Thomsen & Caine, 2011, “Psychomotor stimulant…”).

These two strains also differ in their responses to opioids specifically. Male B6J and A/J mice tested for spontaneous activity after morphine administration display a similar pattern, with high activity in B6J mice and lower activity in A/J mice (Brase, Loh, & Way 1977). The effective dose, or ED50, of chronic morphine is greater in A/J than in B6J, while concentrations of morphine in the brain 30 minutes after injection are the same for both strains (Brase, Loh, & Way 1977; Gill and Boyle 2008). B6J mice also show significantly greater jumping than A/J in response to naloxone-induced morphine withdrawal, suggesting a possible connection between opioid effectiveness and severity of withdrawal (Kest et al. 2009). Susceptibility to common side effects of morphine (constipation and respiratory capacity) are greater in B6J than A/J mice, even though similar doses of morphine yield similar levels of drug in the blood (Young, et al. 2018).

Two tasks were used in the current study to examine behavioral responses to fentanyl. The first was conditioned place preference (CPP), a task which uses conditioning to determine the strength of the rewarding effects of fentanyl. Eight days of conditioning took place,
alternating days between fentanyl in one chamber and saline in the other. Preferences were determined by the proportion of time spent in either a black or white chamber. Final preferences were measured after conditioning and compared to initial preferences. The second behavioral task was a 3-day open field/locomotor activity test, which measured activity for 30 minutes after acute IP injection of either fentanyl or saline on day 1. On day 3, all mice were given saline. This paradigm (from Bryant, Roberts, et al. 2009; Bryant, Chang, et al. 2009) measures acute responses to fentanyl on day 1, and conditioned responses on day 3. Mice that were injected with a drug on day 1 may show conditioned locomotor activity after receiving a saline injection on day 3. Because A/J mice only showed minimal responses to a fentanyl injection at 0.2 mg/kg, two higher doses of fentanyl were also used (0.25 mg/kg and 0.5 mg/kg).

One day after the open field tests, mice were injected with either saline or fentanyl one hour before brain tissue was collected. The tissue was sent to collaborators in the Farber Lab at the University of Virginia, where a characterization of transcriptomic differences was conducted on nucleus accumbens (NAc) tissue using RNA-Seqencing (RNA-seq). This region was chosen because of its key role in the mesolimbic dopamine pathway and addictive behaviors. RNA was returned to NCSU, where qPCR was performed to confirm changes in expression. Two genes were selected from the RNA-seq data set to be confirmed with qPCR- Glyoxalase 1 (Glo1) and Suppressor APC domain containing 1 (Sapcd1). These genes were selected because they showed differential expression between the A/J and B6J strains, and also showed differential expression between saline and fentanyl treatments. Glo1’s role in addictive behaviors has been investigated in many previous studies, which will be discussed later with the results of the current experiment.
Methods

Animals

Male and female adult (>60 days of age) mice from two inbred lines (A/J and B6J) were used to examine differences in gene expression and behavior. All mice were born in the Biological Resources Facility at North Carolina State University, except four A/Js (two males and two females, ordered at 28 days of age from Jackson Labs, Bar Harbor ME). Mice were toe clipped for identification at 7-10 days of age, and were weaned at 21 days of age into groups of two to three mice sharing the same strain, sex, and age. All mice had *ad libitum* access to water and soy-free food (Envigo Teklad 2020, Madison, WI, USA). Lights in the home room were on a 12:12 reverse light schedule. CPP testing was conducted in the light, and open field testing was conducted in the dark portion of the cycle. All animal care and procedures were approved by the NCSU institutional animal care and use committee and are in accordance with AAALAC standards.

Drugs

Mice receiving fentanyl were administered fentanyl HCL at a dose of 0.2 mg/kg via IP injection. This is a moderate dose which has previously been shown to influence behavior in B6J for both tasks used here (Bryant et al 2009). Fentanyl HCL was dissolved in sterile physiological saline, and all injections were administered at a volume of 10 mL/kg. In a final dose-response experiment conducted with A/J mice only, two additional doses of fentanyl (0.25 and 0.5 mg/kg) were used.

Conditioned Place Preference

A/J (n=10 male, n=12 female) and B6J (n=12 male, n=16 female) mice were handled daily for 10 days prior to testing, and were habituated to the testing room for 60 minutes in the two
days prior to testing. The Plexiglas conditioning apparatus consisted of two square (15 cm x 15 cm) chambers with a hole between them. Mice were confined to one side during conditioning using plastic inserts, and were allowed to move freely between the chambers during initial and final preference tests. A clear start box (4.5 x 15 cm) was attached to the outside of both chambers, which was closed after mice left and began the test. A clear plastic lid allowed visibility in both chambers. One chamber consisted of white floors and walls, and the other consisted of black floors and walls. The black chamber contained a plastic covered hard-wire cloth on the floor. These differences gave each side of the chamber unique texture and visual cues (Kudwa et al. 2005). The conditioning apparatus was cleaned with Peroxigard before and after each subject, and chambers were cleaned with 70% ethanol at the end of each day’s tests.

Initial side preferences were determined during a 15-minute recorded test. The initially least preferred side (LPS) was paired with fentanyl injections (0.2 mg/kg ip), and the most preferred side (MPS) was paired with saline. After initial preference was established, eight days of conditioning took place. Conditioning days alternated between fentanyl and saline injections, and fentanyl was always given on the first conditioning day. Conditioning sessions were 30 minutes long and were not recorded. There was a two-day break from conditioning between experimental days 5 and 6. On day 10, final preferences were assessed with a 15-minute free access test. The initial and final preference tests were recorded and analyzed with Noldus EthoVision XT (Leesburg, VA), which tracked the amount of time the subject spent in each chamber.

Open Field Testing

A/J (n=8 male, n=8 female) and B6J (n=8 male, n=8 female) mice were handled daily for three minutes in the three days prior to testing, and were habituated to the behavioral testing room on these days for one hour. Tests were conducted in the dark portion of the light cycle. On
day 1, mice received an injection of saline or fentanyl (0.2 mg/kg IP), and acute locomotor activity was recorded in Open Field (OF) boxes (60 x 60 x 45 cm) for 20 min. The next day all mice were injected with saline and immediately placed back into their home cages. On the third day, all mice received a saline injection and then were placed in the OF and recorded for 20 min. Activity data (cm moved in the horizontal plane) and location (time spent in center) were analyzed for days 1 and 3 using Noldus EthoVision XT (Leesburg, VA).

Higher Doses of Fentanyl in Open Field

Because A/J mice exhibited significantly lower locomotor activity in response to fentanyl, one more round of open field tests was conducted using higher fentanyl doses. We tested an additional four groups of A/J mice (n=8 per group). Each mouse received one of two doses of fentanyl on the first day of the study (either 0.25 or 0.5 mg/kg). All other procedures were identical to those described above for open field testing.

RNA-Sequencing

Mice used in the open field experiment (n=8 per group) were killed 24 hours after the final behavior test. An injection of either saline or fentanyl was given one hour prior to collecting brains to examine changes in gene expression. Mice in the control group (only exposed to saline during testing) received saline, and mice in the fentanyl-treated group received fentanyl (0.2 mg/kg). This injection was given approximately 72 hours after their first day of testing, when mice received their first respective injection. Brains were rapidly removed, and a cold brain dissection mold was used to isolate bilateral nucleus accumbens (NAc) and the medial prefrontal cortex (mPFC). The tissues were then rapidly frozen on dry ice. The NAc was shipped on dry ice to the University of Virginia for RNA-Sequencing, and RNA from these brain regions was later shipped back to NCSU for qPCR.
**Quantitative PCR**

qPCR was conducted using RNA from two brain regions from 32 mice used in the first open-field experiment. Mice were administered either fentanyl (0.2 mg/kg) or saline one hour before brains were extracted. These samples represented B6J and A/J strains, males and females, and fentanyl and saline treatments. The RNA from the NAc and the mPFC was isolated using a Qiagen RNeasy kit, following manufacturers' instructions. cDNA was then generated using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. qPCR was performed using Taqman probes (Table 1) for Sapcd1 and Glo1 (Thermo Fisher). Levels of expression of target genes were normalized to an endogenous control gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). For data evaluation, the comparative ΔΔCt (delta delta Ct) method was used. The gene of interest and the *Gapdh* control were run on the same plates for each sample to eliminate plate-to-plate variation. Samples were run in triplicate for both the gene of interest and *Gapdh*. Samples with Ct values more than two standard deviations from the mean were considered outliers and excluded from the analyses.

**Table 1: TaqMan Probes.** Target genes are shown for each Taqman probe used in qPCR. *Gapdh* was used as a housekeeping/control gene.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Abbreviation</th>
<th>TaqMan Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressor APC domain containing 1</td>
<td>Sapcd1</td>
<td>Mm004584449_m1</td>
</tr>
<tr>
<td>Glyoxalase 1</td>
<td>Glo1</td>
<td>Mm00844954_s1</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Gapdh</td>
<td>Mm99999915_g1</td>
</tr>
</tbody>
</table>
Statistics

CPP data was analyzed by comparing the time spent on each side of the CPP apparatus on the first day (habituation) to the final test day. Two-way ANOVAs were used with sex and strain as the two independent variables. Three dependent measures were analyzed. The initial preference was based on the time spent in the black versus the white chamber of the CPP box during the habituation test. The final preference was calculated by subtracting the time spent on the fentanyl-associated side from the time spent on the saline-associated side on the final test day.

To analyze the open field data, a three-way repeated measures ANOVAs (strain x group x sex) was conducted. The dose-response data were analyzed using a two-way repeated measures ANOVA with dose and sex as the factors. For the qPCR analysis, a three-way ANOVA was used to compare the groups. Planned contrasts were made using Bonferroni-corrected comparisons. All the analyses were conducted using the Number Cruncher Statistical Systems (NCSS) statistical package for windows (NCSS, LLC). Differences were considered statistically significant if the probability of error (p-value) was below 0.05.

Results

*B6J mice, but not A/J mice, form a conditioned place preference for fentanyl.*

During the habituation test, B6J mice did not show a preference for either side of the CPP box, while A/J mice strongly preferred the black side (Figure 1A). This strain difference was significant (F(1,49)=40.02, p<0.00001), but no sex effect was found (F(1,49)=0.13), nor was there an interaction between the two factors (F(1,49)=0.94). Final preference data showed an effect of strain (F(1,49)=31.05, p<0.00001). A/J mice did not form a preference for the
fentanyl-paired chamber, while B6J mice did (p<0.05, Figure 1B). No effect of sex was found for final preference, nor was there any interaction (F(1,49)=0.39 and 0.61 respectively).

Figure 1: Conditioned Place Preference (CPP) for Fentanyl.

Means + SEM for B6J and A/J mice. A) Initial side preferences for black versus white sides of the CPP chamber on the habituation day. B) Differences on the final test day between time spent in the fentanyl-associated versus the saline-associated sides of the CPP chamber.

*Significantly different from time spent on white side for A/J mice, p<0.05. **Significant effect of strain, p<0.00001. We tested Female B6J, n=16, Male B6J, n=12, Female A/J, and Male A/J, n=12. Total test time was 15 minutes. sec=seconds.
B6J mice display increased activity in the open field after acute fentanyl exposure.

Fentanyl-induced activity in the open field and conditioned activity 48 hours later were both greater in B6J than A/J mice (Figure 2). We noted a highly significant effect of strain (F(1,127)= 238.29, p<0.00001), and a significant interaction between strain and treatment (F(1,127)=13.77, p<0.0005) on distance moved during day 1 tests. The interaction was produced by the fentanyl-treated B6J mice, which were significantly more active than all other groups (p<0.05). For all groups, activity on the first test day was significantly higher than the third day (F(1,127)=43.87, p<0.00001). A significant interaction between strain and test day (F(1,127)=93.53, p<0.00001) was noted, likely due to fentanyl-treated mice traveling more than any other group during the first test day (p<0.05).

Strain differences were also apparent on day 3 of the open field test. All mice received a saline injection, so differences between groups that had previously received saline or fentanyl were interpreted as conditioned effects from day 1. We performed a two-way ANOVA for each strain on the data from day 3. In B6J mice, we noted a significant effect of treatment (F(1,31)=9.06, p<0.005) on the distance traveled. Because mice with prior fentanyl experience were more active on this test than the saline-exposed mice, we conclude that their activity was conditioned by the drug exposure during the first test (p<0.05). A/J mice showed no treatment effect on day 3 (F(1,31)=0.48).
Figure 2: Fentanyl-Induced Acute and Conditioned Locomotor Activity

Mean ± SEM horizontal activity in meters. Activity is shown on the left for B6J mice and on the right for A/J mice. The first test day mice received either fentanyl (0.2 mg/kg, striped histograms) or saline (solid histograms) and activity was recorded in the open field (OF). On the third day, all mice received saline prior to the OF test. ***Significant main effect of strain, p<0.00001. **Significantly more activity on day 1 than on day 3, p<0.00001. *Significantly different from fentanyl-treated B6J mice, p<0.05. n=8 per group. Total test time was 20 minutes. M=meters. n=8 per group.

A three-way repeated measures ANOVA revealed a significant strain effect (F(1,56)=129.07, p<0.00001) on location in the OF, with A/J mice spending far less time in the center of the arena than the B6J mice. There was also an interaction between strain and treatment (F(1,56)=5.07, p<0.035) due to the opposite effects of fentanyl between the two strains; B6J mice treated with fentanyl spent less time in the center, and female A/J mice receiving fentanyl increased their
time in the center. We performed two-way ANOVAs for each strain. A sex difference was noted in A/J mice (F(1,28)=4.65, p<0.05), with females spending more time in the center of arena than males. In B6J mice, the two-way ANOVA did not reveal any treatment or sex effects (F(1,28)=2.93, 0.37, respectively).

![Figure 3: Center Time in Open Field](image)

Mean + SEM time spent in the center of the OF. *A/J mice spent less time in the central square than did B6J mice, p<0.00001. **Significantly different than time in center after fentanyl treatment, p<0.05. In A/J mice females spent more time in the center of the arena compared to males, p<0.05. Total test time was 20 minutes, sec=seconds. n=8 per group.
Higher doses of fentanyl did not enhance activity in A/J mice.

Even when A/J mice received more than double the original dose of fentanyl, it had no impact on their activity. A two-way repeated measures ANOVA did not reveal any dose, sex, or interaction effects in A/J mice (F(1,63)=0.16, 0.17 and 0.17 respectively). The only significant effect was a sex by test interaction (F(1,63)=5.14, p<0.03), but Bonferroni-corrected comparisons did not yield any significant differences.

Table 2: Horizontal Activity for A/J Mice. Mean +/- SEM horizontal activity in cm in three day spontaneous and conditioned activity tests. No significant differences were found between groups based on sex or dose. n=8 per group.

<table>
<thead>
<tr>
<th>Fentanyl Dose</th>
<th>Spontaneous Activity (Day 1)</th>
<th>Conditioned Activity (Day 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 mg/kg</td>
<td>915 +/- 130</td>
<td>2,672 +/- 736</td>
</tr>
<tr>
<td>0.5 mg/kg</td>
<td>1,324 +/- 268</td>
<td>3,198 +/- 412</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 mg/kg</td>
<td>826 +/- 151</td>
<td>1,973 +/- 569</td>
</tr>
<tr>
<td>0.5 mg/kg</td>
<td>2,373 +/- 1,461</td>
<td>2,159 +/- 282</td>
</tr>
</tbody>
</table>
Two relevant gene candidate genes were selected from the RNA-seq analysis (conducted by collaborators at UVA) for qPCR- Glo1 and Sapcd1. There was a significant effect of strain on Glo1 expression in the NAc (F(1,30)=1714.13, p<0.00001, Figure 4A), with A/J mice having higher levels of Glo1 mRNA than B6J mice. We noted a trend (F(1,30)=3.59, p=0.071) of a treatment effect and no sex differences (F(1,30)=2.47). Likewise, Sapcd1 was significantly different between the two strains (F(1,29)=19.65, p>0.00025), with A/J mice having higher levels of mRNA than B6J (Figure 4B, p<0.05). No significant differences were noted for treatment (F(1,29)=0.03), although there was a trend for a sex difference (F(1,29)=3.82, p=0.063). Females showed lower Sapcd1 expression in the NAc than males. No interactions were statistically significantly for either gene.

In the mPFC, we noted treatment (F(1,30)=8.61, p<0.008) and strain (F(1,30)=1836.4, p<0.00001, Figure 4C) effects on Glo1 expression. No significant sex differences were found (F(1,30)=2.39). Fentanyl treatment caused lower Glo1 expression in the mPFC (p<0.05). The strain difference, as in the NAc, was driven by higher Glo1 expression in A/J than B6J mPFC (p<0.05). For Sapcd1 mRNA in the mPFC, we noted a large strain effect (F(1,30)=196.62, p<0.00001). Sapcd1 expression was found to be lower in B6J than in A/J PFC (Figure 4D, p<0.05). No significant effects of sex or treatment were found (F(1,30)=0.02, 0.57, respectively).
Figure 4: *Glo1* and *Sapcd1* Expression in NAc and mPFC after Fentanyl Treatment

Mean + SEM relative quantities of mRNA from C57BL/6J and A/J mice. (A) Levels of *Glo1* in the Nucleus Accumbens (NAc). (B) Levels of *Sapcd1* in the NAc, a trend for higher levels in males (p=0.063). (C) Levels of *Glo1* in the mPFC. (D) Levels of *Sapcd1* in the mPFC. *Significant differences between strains, p<0.001, or less. **Significantly less mRNA after fentanyl treatment compared to saline group (p<0.008). n=4-5 per group.

Discussion

A/J and B6J mice displayed significant differences in both behavioral tasks. B6J mice acquired robust place preference for fentanyl during the CPP task, as had been reported previously by Bryant, Roberts, et al. (2009). After conditioning, both male and female B6J mice showed strong preferences for the side of the CPP chamber paired with fentanyl. B6J mice did not show a significant preference for either side during the initial test (Figure 1A). After conditioning, A/J mice did not change their side preference from the initial test, and thus did not
form a preference for the fentanyl-paired side (Figure 1B). Data from A/J mice indicated less of a conditioning effect of fentanyl. One difference to consider is that A/J mice displayed a strong initial preference for the black side of the CPP chamber (Figure 1A). This may be related to higher levels of anxiety in A/J mice compared to B6J (Laarakker, van Lith, & Ohl, 2011), which manifested in the A/J mice spending more time in the darker area. Taken together, these data suggest a stronger conditioning effect of fentanyl on B6J than A/J mice and suggest that B6J mice find fentanyl to be more rewarding.

Strain differences were also apparent in the 3-day open field test (Figure 2). B6J mice were about five times more active than A/J mice in the OF, regardless of treatment. A/J mice were largely inactive for the duration of the test, and administration of fentanyl did not enhance their activity. B6J mice showed a roughly 30% increase in locomotor activity in response to fentanyl. B6J mice who received fentanyl on day 1 showed conditioned locomotor activity on day 3, and were more active than mice that received saline on day 1. Both the reduction in locomotor activity and lack of conditioned activity in A/J mice agree with previous research that suggests a lower overall physiological response to opioids for the A/J strain. Even when higher fentanyl doses (0.25 mg/kg and 0.50 mg/kg) were given to A/J mice, no significant differences were noted, although A/J females showed a trend for higher locomotor activity at the highest fentanyl dose (0.5 mg/kg).

Some of these varying drug responses may be caused by differences in anxiety between the two strains. Female A/J mice that received fentanyl spent slightly more time in the center of the open field on day 1 than other A/J groups, but all A/J mice showed significantly less time in the center compared to B6J mice. Spending less time in the center of the open field suggests higher levels of anxiety in A/J mice, which agrees with previous studies comparing anxiety in A/J and B6J mice (Hovatta et al. 2005; Crawley & Paylor 1997). These data are also consistent with previous research demonstrating lower levels of activity in A/J mice. In a previous study
examining eight inbred mouse strains, A/J mice displayed one of the lowest levels of activity, and B6J mice showed one of the highest. A/J mice also spent less time in the center of the OF than B6J (Solberg et al. 2006). Milner & Crabbe (2008) used the light dark (LD) transition test and found higher activity in B6J mice compared to A/J. In home cage tests, A/J moved less than B6J mice (Solberg et al. 2006). Some studies comparing anxiety in A/J and B6J have shown mixed results. In the elevated plus maze (EPM), a task used to measure anxiety directly, A/J mice spent roughly the same amount of time in the open arms as B6J mice (Moy et al. 2007). Solberg et al. (2006), however, found that A/J mice spent less time in the open portion of elevated zero maze compared to B6J (Milner & Crabbe 2008). Increased anxiety and reduced activity in A/J mice should be considered when interpreting the open field data, as fentanyl-induced activity may be lower in A/J mice solely because of their overall reduced activity compared to B6J.

After the open field test, qPCR was conducted on NAc and mPFC tissue for 2 genes- Glyoxalase 1 (Glo1) and Sapcd1 (Suppressor APC Domain Containing 1). Glo1 has been shown to correlate negatively with anxiety in multiple mouse strains, including A/J and B6J; strains with more Glo1 spend less time in the center of the OF, take longer to enter novel environments, and spend more time in the dark than the light area in the light/dark task. (Hovatta et al. 2005; Williams et al. 2009, Distler et al. 2012). There is a single copy number variation among strains of inbred mice in the Glo1 gene, which leads to variation in expression (Williams et al. 2009). A/J mice have three times the number of gene copies compared with B6J, and comparatively elevated Glo1. The other initially interesting gene, Sapcd1, is associated with the human and mouse MHC region. In humans, this is the most gene-dense region of the genome (Xie et al. 2003). Sapcd1 has associated with a long-non-coding-RNA involving the nearby MSH5 gene (called “MSH5-SAPCD1”), and a SNP in this readthrough RNA was identified in an epidemiological study of schizophrenia (Yamada, et al. 2015. Because
Sapcd1 has less of an established role in behavior, and because qPCR data for Sapcd1 did not confirm treatment difference shown in RNA-seq data, we primarily focused on Glo1.

Glo1 has been identified as contributing to strain differences in behavior, and differences in anxiety in particular. When Glo1 expression is decreased using knockout or knockdown models (or by using a small molecule inhibitor), anxiety is reduced (Hovatta et al. 2005; McMurray et al. 2018; deGuglielmo et al. 2018; Jang et al. 2017). When Glo1 is overexpressed, the reverse occurs (Hovatta et al. 2005; McMurray et al. 2016; Williams et al. 2009). Glyoxalase 1 (Glo1) is an enzyme in the oxoaldehyde metabolic pathway. Glo1 detoxifies methylglyoxal (MG), a byproduct of glycolysis, and the two enzymes have reciprocal actions on a number of behaviors including locomotion and anxiety (Distler & Palmer 2012, Szczepanlk et al. 2020, McMurray et al. 2016). This pathway involving Glyoxalase 1 is also involved with apoptosis, metabolism, and detoxification of reactive oxygen species (Distler & Palmer 2012).

Glo1 was differentially expressed in the mPFC between A/J and B6J strains, and was also differentially expressed between fentanyl and saline treatments. Glo1 was expressed at higher levels in A/J in both the NAc and the mPFC. Fentanyl treatment reduced Glo1 expression in the mPFC, but not the NAc. While both strains showed reduced Glo1 expression after fentanyl treatment, the difference was greater in A/J mice. Recent studies have manipulated Glo1 to examine its effects on alcohol intake in several models, including mice in “drinking in the dark” (McMurray et al. 2017), self-administration in rats (deGuglielmo et al. 2018) and locomotor impairment in mice (Barkley-Levenson, Lagarda, & Palmer 2018). In all three studies, reduced Glo1 activity resulted in decreased alcohol intake. No other studies to our knowledge have examined the relationship between Glo1 and vulnerability to the reinforcing actions of drugs. Although it has been documented in diabetic animal models (Jack, Ryals, & Wright 2011; Williams, et al. 2009) and in diabetic patients (Skapare et al. 2013) that pain is reduced when
Glo1 mRNA is decreased, a connection between Glo1 expression and opioids has not previously been established.

Glo1 is an interesting candidate gene because it directly affects neural signaling. One major mechanism of action of the Glo1 gene is through a reduction in MG in parts of the central nervous system. Because MG is cytotoxic and leads to the formation of reactive oxygen species at high concentrations, the detoxification of MG by Glyoxalase 1 is necessary to prevent cellular damage. MG can act as a partial agonist at the GABA<sub>A</sub> receptor (Distler & Palmer 2012; McMurray et al. 2018), and at high doses, MG decreases dopamine levels in the mPFC (Szczepanik et al. 2020). Although high concentrations of MG can induce apoptosis, previous studies have demonstrated anxiolytic effects in mice within minutes of MG administration, which suggests much more rapid effects (ie. GABAergic effects) than would be expected from cytotoxicity or apoptosis (Distler & Palmer 2012). Studies manipulating Glo1 expression likely influence a pathway where increased Glo1 expression increases anxiety by reducing levels of MG, thereby decreasing GABA<sub>A</sub> receptor activation. Future studies should examine Glo1 and MG concentrations, opioids, and anxiety, the relationship of which has not been investigated previously. Much remains to be understood about MG’s GABAergic actions in the brain.

Gene expression data for Glo1 in the current experiment agreed with previous research suggesting a correlation between Glo1 and anxiety. Glo1 expression was nearly 5-fold higher in A/J mice compared to B6J mice, regardless of treatment. This pattern of expression was consistent in both the NAc and the mPFC. Higher levels of anxiety were reflected in A/J mice by their noticeable lack of time spent in the center of the arena during the open field test. Higher levels of anxiety in A/J mice were also suspected to be the cause of A/J mice showing a strong preference for the black chamber during the initial CPP test. In the NAc, no significant differences were observed between fentanyl and saline treatments, although both strains showed a trend for higher Glo1 expression after fentanyl administration. In the mPFC, fentanyl
administration resulted in significantly reduced Glo1 expression, which confirmed RNA-seq results for the mPFC. These data point to a complex relationship between fentanyl, anxiety, and Glo1 expression. Reductions in Glo1 expression are known to lead to anxiolytic effects, so the fentanyl-induced reduction in Glo1 seen here in the mPFC may partially explain the well-established anxiolytic effects of opioids (Colasanti et al. 2010). Fentanyl is one of the less-studied opioids, so further research should be done to determine the degree of anxiolytic effects induced by fentanyl, compared to other opioids like morphine.

While the NAc (or ventral striatum) is essential to the formation of addictive phenotypes, the mPFC is also a part of the mesolimbic dopamine system, with some dopaminergic neurons projecting from the ventral tegmental area to the mPFC. Research with rats and cocaine has shown associations between GABA-A receptors in the mPFC and neural changes associated with certain elements of addiction. After repeated cocaine exposure, GABA-A-mediated inhibition of the mPFC is reduced, allowing long-term potentiation of excitatory neurons. This process is involved with withdrawal, relapse, and impulsivity- key parts of the addiction cycle (Lu et al. 2010; Huang, Lin, & Hsu 2007; Jupp et al. 2013). Here we propose that Glo1 contributes to strain differences in fentanyl response in A/J and B6J mice, and that fentanyl administration causes changes in Glo1 expression throughout the brain. Because Glo1 has been widely implicated in addiction research with rodents, and because Glyoxalase 1, Methylglyoxal, and GABA-A receptor activity show a clear relationship, we propose that further research investigate Glo1 as a potential therapeutic target for treatment of addictions, including to opioids like fentanyl in particular. Appendix 1 contains preliminary data on fentanyl-induced locomotor activity after administration of pBBG, a Glo1 inhibitor.
References


APPENDIX
Appendix 1: pBBG and Fentanyl in the Open Field

To determine whether Glo1 is involved with the differences in fentanyl response between A/J and B6J mice, we conducted an additional round of open-field testing with fentanyl and S-bromobenzylglutathione cyclopentyl diester (pBBG). This compound has been used previously as a Glo1 inhibitor to investigate the role of Glo1 in ethanol self-administration and sensitivity in mice (McMurray et al. 2017). No previous work has used pBBG to establish a connection between Glo1 and sensitivity to opioids. Here we investigated whether pretreatment of mice with pBBG two hours prior to an open field task would modulate locomotor activity after fentanyl or saline injection. Because B6J mice showed greater responses in all of the previous tasks, only B6J mice were used for this additional open field task.

As discussed previously, administration of pBBG reduced mouse ethanol consumption in multiple studies. In a locomotor activity task with ethanol, pBBG (7.5 mg/kg) has been shown to significantly reduce locomotor activity at multiple ethanol doses, and to slightly increase locomotor activity in the control (zero ethanol) group (Barkley-Levenson, Lagarda, & Palmer 2018). This is an interesting result when considering Glo1 as a potential therapeutic target for addiction. The effects of pBBG (or other modulators of Glo1 expression) on behavior may depend on other drugs being ingested at the same time. When pBBG (10 mg/kg) was administered for five days to multiple mouse strains, BDNF was significantly upregulated in the mPFC and the hippocampus. An increase in BDNF is consistent with an antidepressant effect (McMurray et al. 2018). To confirm the effects of pBBG on behavior, researchers can administer MG directly to mice. Because the effect of pBBG administration is a reduction in Glo1 activity, pBBG would be expected to increase levels of MG, and thus have similar effects to MG administration itself. Szczepanik et al. (2020) treated Swiss mice with MG and found a significant reduction in locomotor activity at higher, but not lower doses. Anxiolytic effects were observed in an open field task four hours after MG administration.
pBBG used in the current locomotor activity tests was obtained from the Barkley-Levenson lab (Department of Psychiatry, University of California San Diego). pBBG was dissolved in a vehicle composed of 8% DMSO, 18% Tween-80, and 74% saline. Injections were given intraperitoneally at a volume of 5 mL/kg. Two hours prior to the locomotor activity tests, mice were administered either vehicle or pBBG (12.5 or 25 mg/kg). Mice were administered fentanyl HCL at a dose of 0.2 mg/kg via IP injection. Fentanyl HCL was dissolved in sterile physiological saline, and all injections were administered at a volume of 10 mL/kg.

Male (n=43) and female (n=35) B6J mice were assigned to one of six groups: fentanyl and low-dose pBBG (n=13), fentanyl and high-dose pBBG (n=11), fentanyl and vehicle (n=16), saline and low-dose pBBG (n=15), saline and high-dose pBBG (n=7), or saline and vehicle (n=16). Mice were 8-12 weeks of age at the time of testing. The open field task was conducted in a slightly different manner than described previously. Mice in the current open field experiment were first injected with either vehicle or one of two pBBG doses, and were returned to their home cages for one hour and 40 minutes. Then, mice were removed and placed into the open field for 20 minutes. This portion was not recorded, and was meant to habituate mice to the open field. After 20 minutes, mice were picked up from the open field, injected with either fentanyl (0.2 mg/kg) or saline, and placed back into the center of the open field. Recording then began for 30 minutes. This round of open field tests was conducted in the light portion of the light cycle.

To analyze the open field data, a three-way repeated measures ANOVAs (strain x group x sex) was conducted. There was a significant effect of fentanyl treatment on day 1. Fentanyl treatment caused significantly increased locomotor activity (F(1,77)= 73.8, p<0.00001) and center time (F(1,77)=16.7, p<0.001). Reductions in locomotor activity after pBBG treatment were greater for the 12.5 mg/kg pBBG group than the 25 mg/kg group, but no significant effects of pBBG treatment were shown in the repeated measures ANOVA for either group. A weak
trend was found for an interaction effect between the two injections (Injection 1 = pBBG or vehicle, Injection 2 = saline or fentanyl) for distance moved (F(1,77)=1.27, p=0.287). No significant differences between males and females were noted for any group.

![Distance Moved in Open Field (Day 1) after treatment with Fentanyl and pBBG](image)

**Figure 5: Distance Moved in Open Field (Day 1) after treatment with Fentanyl and pBBG**

Mean + SEM distance Moved during a 30-minute open field test after injection with either low-dose (12.5 mg/kg) pBBG, high-dose (25 mg/kg) pBBG, or vehicle. *Mice receiving low-dose pBBG and fentanyl showed a significantly lower distance moved than mice receiving vehicle and fentanyl (p<0.05).

Figure 5 shows the distance moved by each group on day 1. Groups receiving fentanyl showed substantially higher locomotor activity than all groups receiving saline. pBBG treatment did not emerge as a significant factor in the three-way ANOVA that was conducted, although the dose-dependent response of pBBG may have obscured its effects in the ANOVA. Because the
3-way ANOVA was the primary statistical test run and no significant effects of pBBG treatment were found, the pBBG OF experiment is included as an appendix, rather than in the body of the paper. However, an independent samples t-test showed a significant difference in locomotor activity on day 1 between the low-dose (12.5 mg/kg) pBBG and the vehicle groups that received fentanyl (t(27)= -2.072, p=0.0480). Interestingly, the reduction in fentanyl-induced locomotor activity was much greater for the low-dose pBBG group than for the high-dose group, which may explain partially why no significant effect of pBBG was seen in the three-way ANOVA.

Low-dose pBBG treatment resulted in a significant reduction in distance moved on day 1 only for mice receiving fentanyl. Mice receiving saline showed similar levels of locomotor activity regardless of pBBG treatment. This surprising result requires more research to fully explain, but suggests that the Glo1 inhibitor pBBG may reduce sensitivity to the behavioral effects of opioids. Recall that many previous studies have established a link between behavioral sensitivity to a drug and the strength of the rewarding/reinforcing effects. Because pBBG (12.5 mg/kg) reduces fentanyl-induced locomotor activity, future studies should investigate whether pBBG can reduce self-administration or conditioned place preference formation in response to fentanyl. Because this experiment is preliminary, Glo1 expression was not quantified after pBBG administration. Future experiments should measure Glo1 expression in multiple brain regions after exposure to a range of pBBG doses.