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Article

Digestion products of bread and cheese cause addictive behavior in a planaria model

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SUMMARY

Addiction is defined as compulsive engagement in certain practices despite considerable danger and other harmful consequences. Gluteomorphin and casomorphin are peptides derived from the digestion of bread and cheese and are known to bind to opioid receptors. However, it is unclear if gluteomorphin or casomorphin are addictive in a similar manner to extrinsic opioids such as morphine. Since 75% of the American diet consists of wheat and dairy, the implication of addiction could have a major impact on diet and obesity. Thus, the purpose of this study was to investigate the addictive potential of gluteomorphin and casomorphin in a planaria model. We conducted controlled experiments comparing planaria exposed to water, gluteomorphin, or casomorphin. We utilized light/dark testing, motility, & stereotypical movements to test for acute exposure, withdrawal, sensitivity/tolerance, environmentally placed conditioning, and cross sensitivity with nicotine. Our results from acute exposure, abstinence induced withdrawal, environmentally placed conditioning, and sensitization/tolerance assays suggest that gluteomorphin and casomorphin are addictive. Additionally, results in cross sensitization testing with nicotine were inconclusive and further evaluation is recommended. These findings establish grounds to further investigate the potential addictiveness of gluteomorphin and casomorphin, digestion products of bread and cheese respectively.

INTRODUCTION

Opioids are peptide molecules composed of 5–80 amino acids. Opioids activate by binding to opioid receptors, located mostly on nerve cell membranes (1). Activation of opioid receptors produce feelings of pleasure and pain relief, which can lead to dependence and addiction (2). Opioid addiction is defined by a compulsive urge to continue to take opioids even if unnecessary (3). Opioid dependence is defined by the occurrence of physical and emotional symptoms when deprived of the opioids (3).

Gluteomorphin and casomorphin are peptides derived from the digestion of bread and cheese and are known to bind to opioid receptors (4). Because the digestion of bread and cheese produces biologically active peptides that bind to opioid receptors, it is possible that eating bread and cheese may result in addictive-like behavior. This investigation is warranted because 75% of the American diet consists of wheat and dairy, as well as the fact that two-thirds of the American population is obese (5). The implication that bread and cheese can cause addictive-like behavior could have major consequences for dieting and obesity. Obese people tend to report craving food in the absence of hunger (6).

Flatworms known as planaria are cost effective model organisms that have been used previously in drug addiction studies involving cocaine, nicotine, ethanol, as well as other opioids (7, 8). This study examined *Dugesia dorotocephala* planaria, flatworms that absorb drugs through the skin. Planaria work as fitting model organisms for this experiment because they have neurotransmitter systems comparable to mammals that are impacted by addictive substances. Planaria are considered a good model organism for addiction behavior investigations because they have a central nervous system and use the same neurotransmitters as humans such as serotonin, γ-aminobutyric acid, dopamine, glutamate, and acetylcholine; dopamine and glutamate are known to play a role in human opioid addiction (9, 10). Planaria also demonstrate similar behaviors to addictive substances as humans. These behaviors include altered motility and stereotypical movements with short term exposure to addictive substances, indications of anxiety upon abstinence induced withdrawal as indicated by environmental place conditioning or changes in preference for light and dark environments, sensitization or tolerance to a drug, and cross sensitization (11).

Thus, the purpose of this study was to determine if gluteomorphin or casomorphin can cause addictive-like behavior in a planaria model using established behavioral assays. In our study, we found that gluteomorphin and casomorphin can cause addictive behavior in a planaria model, suggesting the need for additional research investigating potential addictive qualities of bread and cheese products for humans.

RESULTS

To study the addictive potential of gluteomorphin and casomorphin on planaria, we performed six established behavioral assays utilizing 114 uM concentrations of gluteomorphin and 173 uM concentrations of casomorphin, which we compared to a water control. We chose these concentrations because similar concentrations were used for other addictive substances including an antidepressant and a stimulant drug (12).

Acute exposure decreased motility and increased stereotypical movements

To assess the effect of acute exposure to gluteomorphin or casomorphin, we exposed planaria to each of these substances or to water for five minutes and counted the number of C-like hyperkinesia (stereotypical movements) and

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the number of grid lines crossed by planaria. Acute exposure assay results showed planaria exposed to casomorphin displayed decreased motility compared to a water control with an average of 58 grid line crosses compared to 110 grid line crosses for the water control ($p < 0.00001$, **Figure 1**). Planaria exposed to gluteomorphin displayed decreased motility compared to a water control with an average of 63 grid line crosses compared to 110 grid line crosses ($p = 0.000028$, **Figure 1**). In addition, planaria exposed to casomorphin had increased stereotypical movements compared to a water control with an average of 5 C-like hyperkinesia compared to 0.1 C-like hyperkinesia for water ($p = .001616$, **Figure 1**). Planaria exposed to gluteomorphin also had increased stereotypical movements compared to a water control with an average of 6 C-like hyperkinesia compared to 0.1 C-like hyperkinesia for water ($p = 0.000117$, **Figure 1**).

Abstinence induced withdrawal resulted in decreased motility

To test the effect of removal from gluteomorphin or casomorphin, planaria were kept in gluteomorphin, casomorphin, or water for 60 minutes and then were returned to Petri dishes containing water. After, the number of times planaria crossed grid lines was counted. Planaria exposed to casomorphin displayed decreased motility compared to a water control with an average of 16 grid line crosses compared to 115 grid line crosses for water ($p < 0.00001$, **Figure 2**). Planaria exposed to gluteomorphin also displayed decreased motility compared to a water control with an average of 25 grid line crosses compared to 115 grid line crosses for water ($p < 0.00001$, **Figure 2**).

Abstinence induced withdrawal decreased time spent in the light

To test the effect of removal from gluteomorphin or casomorphin using a second method, planaria were kept in gluteomorphin, casomorphin or water for 60 minutes and then returned to Petri dishes containing water with half of the dish exposed to light and the other half of the dish exposed to dark. This time frame was based on a previously established experiment testing for abstinence in planaria where scientists varied their exposure time from 60 minutes to 30 minutes and so on, progressively decreasing (9, 13). Due to the setting and timeframe of this experiment, 24 hours was unreasonable so the next most effective time frame (60 mins) was used. The amount of time planaria spent in the light was recorded for five minutes. Planaria exposed to casomorphin displayed decreased time spent in the light compared to a water control with an average of 27 seconds in the light compared to 86 seconds in the light ($p = 0.00879$, **Figure 2**). Planaria exposed to gluteomorphin displayed similarly decreased time spent in the light compared to a water control with an average of 25 seconds in the light compared to 86 seconds in the light ($p = 0.007082$, **Figure 2**).

Planaria spent more time in their least preferred area

In a pre-test, planaria were placed in a half-light, half-dark Petri dish with water for five minutes. Since all planaria spent less time in the light, it was defined as the least preferred environment (data not shown). Thus, we expected that planaria who were exposed to drugs in their least preferred environment would continue to seek their least preferred environment.

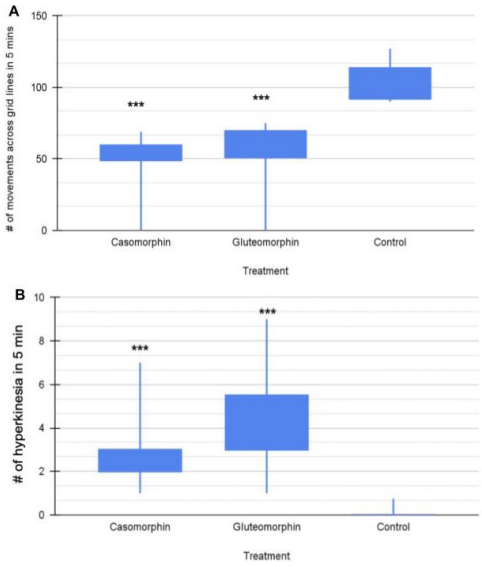

Figure 1: Acute exposure to casomorphin and gluteomorphin on planaria. Planaria were exposed to a 173uM concentration of casomorphin (n=10) or a 114uM concentration of gluteomorphin (n=10) for 5 minutes. **A)** Motility and **B)** hyperkinsesia were measured after exposure. Differences between the casomorphin group and the control group and the gluteomorphin group and the control group are significant for both the motility and hyperkinesia tests, ***p<0.001 according to the Bonferroni correction test with an adjusted significance level of 0.025. Error bars present 95% standard deviation.

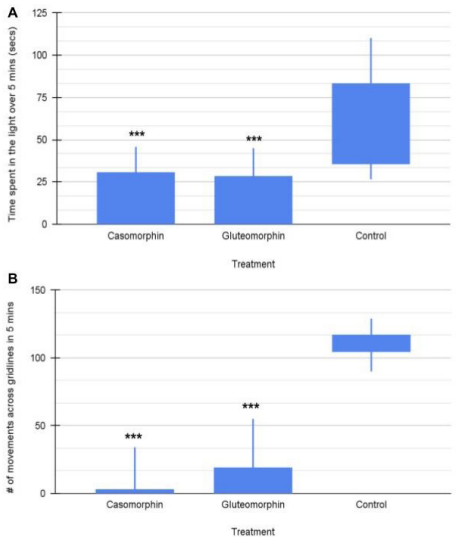

Figure 2: Effect of abstinence from casomorphin and gluteomorphin on planaria. Planaria were exposed to a 173uM concentration of casomorphin (n=10) or a 114uM concentration of gluteomorphin (n=10) for 60 minutes; planaria then placed into a half-covered Petri dish with water for 5 minutes. **A)** Time spent in the light and **B)** motility were measured after exposure. Differences between the casomorphin group and the control group and the gluteomorphin group and the control group are significant for both the motility and light/dark tests, ***p < 0.001 according to the Bonferroni correction test with an adjusted significance level of 0.025. Error bars present 95% standard deviation.

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After exposure to each drug or water, the amount of time planaria spent in their least preferred environment, the light, was recorded over 5 minutes. Overall, drug-exposed planaria spent more time in the light. Planaria exposed to casomorphin displayed increased time spent in the light compared to a water control with an average of 147 seconds compared to 80 seconds in the light ($p = 0.039911$, **Figure 3**). Planaria exposed to gluteomorphin also displayed increased time spent in the light compared to a water control with an average of 155 seconds compared to 80 seconds in the light ($p = 0.02798$, **Figure 3**).

Sensitivity/tolerance assays accentuated decreased motility and increased stereotypical movements

To test for sensitivity or tolerance to gluteomorphin or casomorphin, planaria were exposed three times over four days to either casomorphin, gluteomorphin, or water. The number of grid lines crossed and C-like hyperkinesia were counted for each exposure. Planaria exposed to gluteomorphin showed a continuous decrease in motility from day 1 exposure with an average of 62.9 crosses compared to day 4 exposure, which had an average of 0.4 crosses ($p < 0.00001$, **Figure 4**). In addition, planaria exposed to gluteomorphin showed increased C-like hyperkinesia from day 1 exposure with an average of 5.5 C-like hyperkinesia compared to day 4, which had an average of 9.2 C-like hyperkinesia ($p < 0.00001$, **Figure 4**). Planaria exposed to casomorphin also showed a continuous decrease in motility from day 1 exposure with an average of 56.4 crosses compared to day 4 exposure, which had an average of 0.5 crosses ($p < 0.00001$, **Figure 4**). Planaria exposed to casomorphin also showed increased C-like hyperkinesia from day 1 exposure with an average of 5.0 C-like hyperkinesia compared to day 4, which had an average of 9.3 C-like hyperkinesia ($p = 0.0016$, **Figure 4**). Planaria exposed to the water control did not show a continuous decrease in motility from day 1 exposure with an average of 115.6 crosses compared to day 4 exposure, which had an average of 115.4 crosses (**Figure 4**). Planaria exposed to the water control had an average of 0.5 hyperkinesia on day 1 and an average of 0.0 C-like hyperkinesia on day 4 (**Figure 4**).

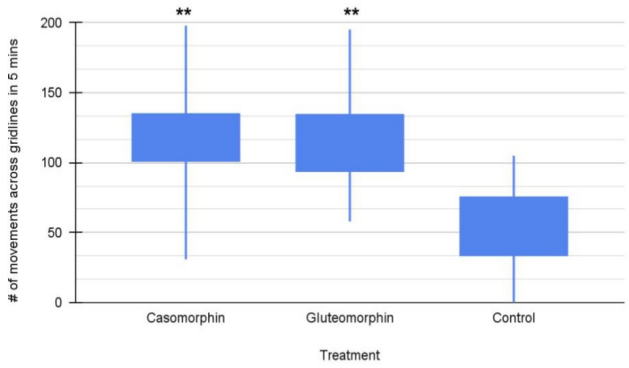

Figure 3: Exposure to casomorphin or gluteomorphin causes environmentally placed conditioning. Planaria were exposed to a 173uM concentration of casomorphin (n=10) and a 114uM concentration of gluteomorphin (n=10) for 60 minutes in the light, the least preferred environment, then put back into the water for a 5 minute test. Motility was measured after exposure. Differences between the casomorphin group and the control group and the gluteomorphin group and the control group are significant, $^{**}p < 0.01$ according to the Bonferroni correction test with an adjusted significance level of 0.025. Error bars present 95% standard deviation.

Cross sensitization with nicotine increased motility and stereotypical movements

Nicotine is an extremely common addictive drug used by people around the world. In combination with other opioids, nicotine can enhance the effect of other drugs and magnify addictive behaviors (9). To determine if nicotine exposure increased sensitivity to gluteomorphin or casomorphin, planaria were exposed twice to 0.1% nicotine on day 1 and then exposed to gluteomorphin, casomorphin, or water on day 4. The assay came from an established experiment using nicotine, and the same timelines and procedures were utilized (14). The number of times planaria crossed gridlines and the number of C-like hyperkinesia were counted. Planaria exposed to gluteomorphin showed a continuous increase in motility from day 1 exposure with an average of 0.6 crosses compared to day 4 exposure, which had an average of 41.6 crosses ($p < 0.0001$ compared to day 4 control, **Figure 5**). In addition, planaria exposed to gluteomorphin showed increased C-like hyperkinesia from day 1 exposure with an average of 3.3 C-like hyperkinesia compared to day 4, which had an average of 9.1 C-like hyperkinesia ($p < 0.0001$ compared to day 4 control, **Figure 6**). Planaria exposed to casomorphin also showed a continuous increase in motility from day 1 exposure with an

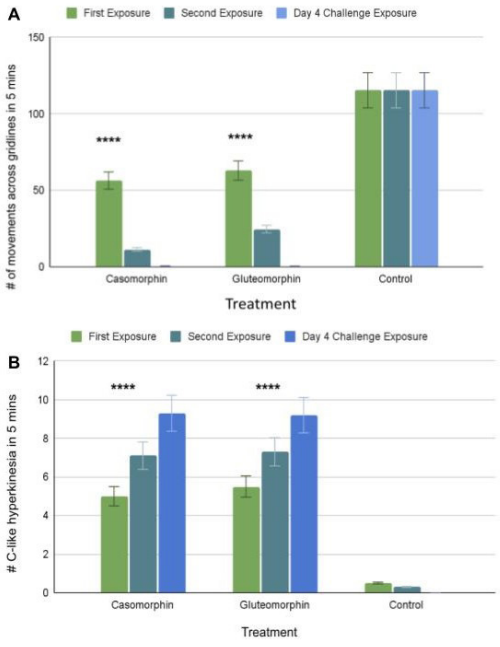

Figure 4: Planaria show sensitization to casomorphin and gluteomorphin. Planaria were exposed to a 173uM concentration of casomorphin (n=10) or a 114-uM concentration of gluteomorphin (n=10) for 5 minutes, three times over the span of 4 days. **A)** Decreased motility and **B) i**ncreased stereotypical movements were measured after exposure. Differences between the casomorphin group and the control group and the gluteomorphin group and the control group are significant for both the motility and hyperkinesia tests, $^{****}p<0.0001$ according to the Bonferroni correction test with an adjusted significance level of 0.025. Error bars present 95% standard deviation.

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average of 0.4 crosses compared to day 4 exposure, which had an average of 54.2 crosses ($p < 0.0001$ compared to day 4 control, **Figure 5**). Planaria exposed to casomorphin also showed increased C-like hyperkinesia from day 1 exposure with an average of 3.4 C-like hyperkinesia compared to day 4, which had an average of 9.2 C-like hyperkinesia ($p < 0.0001$ compared to day 4 control, **Figure 6**). Planaria exposed to the water control did not show a continuous decrease in motility from day 1 exposure with an average of 115.5 crosses compared to day 4 exposure, which had an average of 116.2 crosses (**Figure 5**). Planaria exposed to the water control had an average of 0.5 hyperkinesia on day 1 and an average of 0.0 C-like hyperkinesia on day 4 (**Figure 6**).

Overall, planaria exposed to gluteomorphin or casomorphin showed decreased motility and increased stereotypical movements with acute exposure, decreased motility and time spent in the light with abstinence induced withdrawal, and environmental place conditioning compared to a water control. Our results also displayed increased drug sensitivity due to an accentuation of decreased motility and increased stereotypical movements. These results suggest that both substances are potentially addictive. Cross sensitization results were inconclusive.

DISCUSSION

We hypothesized that digestion products of bread and cheese, gluteomorphin and casomorphin, could be addictive due to their ability to bind to opioid receptors. The results of this study provided evidence suggesting that gluteomorphin and casomorphin produce behavioral alterations in planaria consistent with addictive behaviors similar to mammals. These behaviors included decreased motility and increased stereotypical movements with acute exposure, decreased motility and decreased time spent in the light upon withdrawal, increased time spent in the planaria's least preferred area upon conditioning, and sensitization to gluteomorphin and casomorphin upon repeated exposure. Results from the cross sensitization with nicotine test were inconclusive.

Reduced motility has been established as evidence of addiction in planaria (8). Previous studies with nicotine, a stimulant, demonstrated increased motility with low concentrations and decreased motility with high concentrations (8). The results of our study demonstrated decreased motility with acute exposure to gluteomorphin and casomorphin, which was inconsistent with a previous study using the synthetic opioid [D-Ala2, NMePhe4, Gly-ol5]enkephalin (DAMGO) (9). Specifically, we decided to compare our results to DAMGO because it is a known opioid and thus can act as a beneficial comparison. Since DAMGO is specific to mu opioid receptors and it is known that the kappa opioid receptor causes decreased motility, it is possible that gluteomorphin and casomorphin bind to kappa opioid receptors and not mu opioid receptors (9). In addition, a hallmark of substance abuse is stereotypical movements, a repetitive and purposeless type of movement (9). The increase in the number of C-shape hyperkinesia (a type of stereotypical movement in planaria) was increased upon acute exposure, which was consistent with previous studies using nicotine and DAMGO (15, 8). The water control showed no alteration in motility or stereotypical movements.

Physical dependence upon withdrawal is a prominent characteristic of addiction (9). In previous studies, planaria subjected to opioids, amphetamines, or cocaine displayed decreased motility upon withdrawal from these substances (15). This decrease in motility is a depression or anxiety-like behavior (16). The results for gluteomorphin and casomorphin were consistent with these previous studies displaying decreased motility. The water control showed no alteration in motility. A second method for measuring withdrawal, the light-dark assay, resulted in decreased time spent in the light compared to a water control. Decreased time spent in the light was consistent with previous experiments with DAMGO

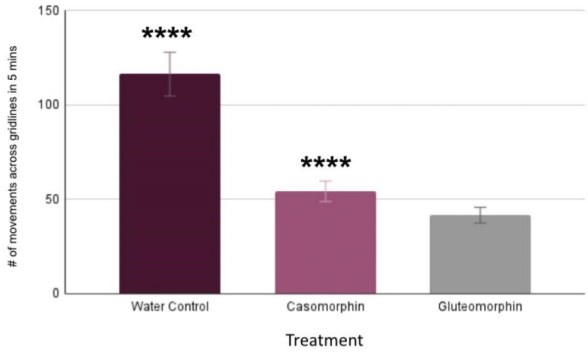

Figure 5: Decreased motility on day 4 may suggest that casomorphin and gluteomorphin display cross sensitization with nicotine. Planaria were exposed to a 0.01% solution of nicotine for 5 minutes on day 1, two times. Then, the same planaria were exposed to a 173uM concentration of casomorphin (n=10) or a 114uM concentration of gluteomorphin (n=10) for five minutes on day 4. Motility was measured after exposure. Differences between the casomorphin group and the control group and the gluteomorphin group and the control group are significant for both the motility and hyperkinesia tests, ****$p < 0.0001$ according to the Bonferroni correction test with an adjusted significance level of 0.025. Error bars present 95% standard deviation.

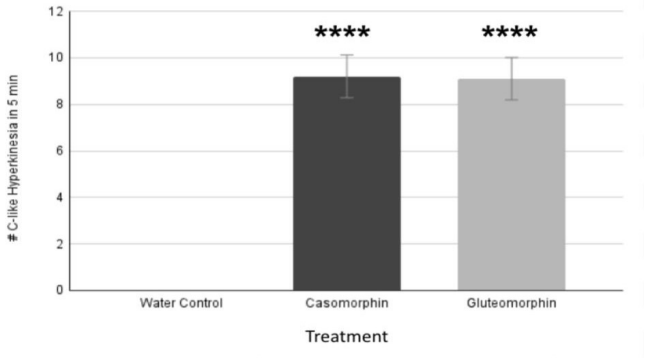

Figure 6: Increased hyperkinesia on day 4 may suggest that casomorphin and gluteomorphin display cross sensitization with nicotine. Mean number of C-like hyperkinesia in 5 minutes. Planaria were exposed to a 0.01% solution of nicotine for 5 minutes on day 1, two times. Then, the same planaria were exposed to a 173-uM concentration of casomorphin (n=10) or a 114-uM concentration of gluteomorphin (n=10) for 5 minutes on day 4. # C-like hyperkinesia was measured after exposure. Differences between the casomorphin group and the control group and the gluteomorphin group and the control group are significant, **** $p < 0.0001$ according to the Bonferroni correction test with an adjusted significance level of 0.025. Error bars present 95% standard deviation.

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(13). The normal behavior of planaria demonstrates negative phototaxis, an avoidance of light. This behavior is considered a defense response related to anxiety (9). The exaggerated decreased time spent in the light is considered to be a defense response to anxiety, a common response to withdrawal when drug dependent (13). A limitation of this test is that we could not rule out the possibility of decreased motility affecting the behavior of the planaria.

Gluteomorphin and casomorphin were paired with light, the least preferred environment for planaria. The idea was to determine if planaria would display drug seeking behavior typical of addicts by spending more time in the light after being conditioned; the light side was perceived as the reward if the planaria were addicted to casomorphin or gluteomorphin. Consistent with previous studies with DAMGO (5), planaria conditioned with gluteomorphin or casomorphin spent more time in the light compared to a control. This behavior could potentially be due to anxiety as well, and this study was unable to determine if the planarian behavior was in fact drug seeking or anxiety related. However, increased time spent in the light is indicative of addiction.

We hypothesized that if repeated exposure to gluteomorphin or casomorphin caused increased sensitivity, then there should be an accentuation of decreased motility and an accentuation of increased stereotypical movements from day one to day four. Further, if repeated exposure to gluteomorphin or casomorphin causes increased tolerance, then there should be an accentuation of increased motility and an accentuation of decreased stereotypical movements from day one to day four. The results demonstrated an accentuation of decreased motility and an accentuation of increased stereotypical movements from day one to day four compared to the water control, suggesting that gluteomorphin and casomorphin caused increased drug sensitivity. No known sensitization studies were done with opioids in planaria, but planaria exposed to cocaine in previous studies produced behavioral sensitization with enhanced activity counts on day four compared to day one (9). Since cocaine is a stimulant, it is expected that they would have the opposite effect, which was increased motility rather than decreased motility.

A previous study on nicotine showed that nicotine can cause alterations in opioid receptors, in particular kappa opioid receptors, which heightens the addictive properties of nicotine (8). We hypothesized that planaria exposed to nicotine, a commonly used drug, prior to exposure to gluteomorphin or casomorphin would cause increased sensitivity to gluteomorphin or casomorphin. The results showed an increase in motility and hyperkinesia. Two issues were raised with this result. First, based on previous results from this study, the expectation was that motility would decrease. A previous study on nicotine showed that nicotine can cause alterations in opioid receptors, in particular kappa opioid receptors, which heightens the addictive properties of nicotine (17). Perhaps, this was responsible for the increased motility. The second issue was that this study only compared the effect of nicotine exposure followed by either gluteomorphin, casomorphin, or a water control. The study did not include a control of no nicotine exposure. Therefore, our results in the cross sensitization with nicotine test were inconclusive and further evaluation is recommended.

The results of this study may have important implications to diet and obesity. Obesity has become an increasingly important health concern (10). Obesity is considered a significant risk factor for diabetes, cardiovascular disease, coronary heart disease, stroke, gastrointestinal disorders, respiratory disorders, musculoskeletal disorders, cancer, psychosocial issues, and overall increase in mortality (10). Additionally, there has been previous research that suggests that the intake of energy-dense foods can change the brain's reward pathway that is associated with the establishment of drug addiction, and that obese individuals demonstrate similar eating patterns to addicted individuals in their consumption of drugs (18). If in fact gluteomorphin and casomorphin, digestion products of bread and cheese, are addictive, it becomes imperative to treat diet as an addiction when treating obesity and create new methods of dieting or treatments.

The results of this study could have been impacted by inability to synchronize planaria ages (due to time limitations), reuse of gluteomorphin and casomorphin for each assay (due to school budget limitations) that may have resulted in changes in concentration, and a lack of a control with no nicotine in the cross-sensitization assay. Also, our results could have been impacted by differences between the pure version of casomorphin and gluteomorphin compared to the ones produced in human bodies, and differing ways of drug intake between the planaria and humans. Additionally, our results could have been affected by different concentrations of gluteomorphin and casomorphin used in the experiment compared to those in human diets, and humans being exposed to casomorphin and gluteomorphin at a different time than in the experiment. Future studies could seek to definitively identify the kappa opioid receptor as the target for gluteomorphin and casomorphin, develop an assay to distinguish drug seeking behavior from anxiety, study the effects of different concentrations of gluteomorphin and casomorphin on planaria addictive behavior, and further elucidate the interactions between nicotine, gluteomorphin, and casomorphin. Behavioral assays were important to complete as a baseline to assess whether the substances are addictive. To supplement these conclusions, we could also complete various "omics" studies (i.e. transcriptomics or proteomics) to better understand the processes the planaria actually underwent to present addictive behaviors.

In conclusion, our study found that the digestive products of bread and cheese are potentially addictive in a planaria model, indicating a need for additional studies in humans and possible implications for diet and obesity.

METHODS AND MATERIALS

Dugesia dorotocephala (planaria) were obtained from Carolina Biological, kept in a plastic tank filled with Poland Spring water under dim light at room temperature, and were fed beef liver once a week. Five milligrams of casomorphin hydrochloride (Sigma-Aldrich) was used to make a 100 ml final concentration of 10mM solution, and 5mg of gluteomorphin (gliadorphin-7; CPC Scientific) was used to make 100 ml final concentration of 10 mM solution. Nicotine in a 0.01% solution was obtained from Carolina Biological. All solutions were prepared with Poland Spring water.

Five types of previously published methods for behavioral assays regarding addiction were performed on planaria exposed to gluteomorphin, casomorphin, or a water control: acute exposure assays, abstinence induced withdrawal assays, environmental place conditioning assays, sensitivity/

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tolerance assays, and cross sensitivity assays (8, 9, 19). Ten trials were performed for each assay and all timelines were based on established protocols for each behavioral assay. Poland Spring water was used as a control for all.

Acute exposure assays

The first test assessed impact on motility. Planaria were placed in a 60 mm Petri dish filled with either 10 ml of 10 mM gluteomorphin solution, 10 ml of 10 mM casomorphin solution, or 10 ml of spring water. Petri dishes were placed over standard graph paper with gridlines spaced 5 mm apart. The number of crosses over the gridlines were recorded over a 5-minute period. The second test assessed stereotypical movements of addiction. Using the same set up as the motility method, hyperkinesias were measured by counting the number of times the planaria stopped moving and curled up into a C-like shape during a 5-minute time period.

Abstinence induced withdrawal assays

The first test assessed impact on motility. Planaria were pretreated in either a 60 mm Petri dish filled with 10 ml of the 10 mM gluteomorphin solution, 10 ml of the 10mM casomorphin solution, or 10 ml of spring water for 60 minutes. Each group of planaria was then placed into Petri dishes with 10 ml of water for 5 minutes following pretreatment. Petri dishes were then placed over standard graph paper and the number of crosses over the gridlines was recorded over a 5-minute period. The second test assessed reactions to light vs. dark environments. Planaria were pretreated in the same manner as the motility method for 60 minutes, then placed in 60 mm Petri dishes filled with 10 ml of water. The Petri dishes were set up with construction paper, with one completely dark side and one side exposed to the light. Planaria were placed on the midline of the Petri dish and the amount of time spent in the light during a 5-minute period was recorded.

Environmentally placed conditioning assay

As a pre-test, planaria were placed in a half-light half-dark 60 mm Petri dish constructed the same way as the light/dark test with 10 ml of water for 5 minutes. The amount of time spent in the light was recorded. Whichever side the planaria spent less time in was defined as the least preferred environment. To condition, planaria were placed in a 60 mm Petri dish with either 10 ml of the 10 mM gluteomorphin solution, 10 ml of the 10 mM casomorphin solution, or 10 ml of spring water; the Petri dish along with the planaria was then placed in the least preferred environment according to the pretest for 60 minutes. The post-test was performed in the same way as the pre-test.

Sensitivity/tolerance assay

Planaria were placed in a 60 mm Petri dish filled with 10 ml of the 10 mM gluteomorphin solution, 10 ml of the 10 mM casomorphin solution, or 10 ml of spring water. Petri dishes were placed over standard graph paper and after five minutes the number of crosses over the gridlines were recorded for a 5-minute period. Each group was then placed in Petri dishes with 10 ml of spring water. This process was repeated 60 minutes later and 3 days later.

Cross sensitization assays

The first test assessed motility. Planaria were placed in three 60 mm Petri dishes each filled with 10 ml of 0.1% nicotine solution. The Petri dishes were placed over standard graph paper and after five minutes the number of crosses over the gridlines were recorded for a 5-minute period. Each group was then placed in Petri dishes with 10 ml of spring water. This process was repeated 60 minutes later. On day four, the planaria exposed to nicotine were placed either in 10 mM gluteomorphin, 10 mM casomorphin, or water. Petri dishes were placed over standard graph paper and after five minutes the number of crosses over the gridlines were recorded for a 5-minute period. The second test assessed stereotypical movements of addiction. Performed the same way as the motility assay but instead of counting crosses over gridlines, the number of times the planaria stopped moving and curled up into a C-like shape was counted.

Statistical analysis

To correct for multiple comparisons, we used the Bonferroni correction test to compare multiple tests within the same set of independent data (i.e. casomorphin vs. control and gluteomorphin vs. control). We divided our alpha level of 0.05 by two, and all of our assays were then compared to a p-value threshold of 0.025 to determine statistical significance.

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REFERENCES

1. Wang, Yulin, et al. “In Vivo Trafficking of Endogenous Opioid Receptors.” *Life Sciences*, vol. 83, no. 21–22, 5 Oct. 2008, pp. 693–99, https://doi.org/10.1016/j.lfs.2008.09.023.
2. Machelska, Halina, and Melih Celik. “Opioid Receptors in Immune and Glial Cells—Implications for Pain Control.” *Frontiers in Immunology*, vol. 11, no. 300, 4 Mar. 2020, https://doi.org/10.3389/fimmu.2020.00300.
3. Sussman, Steve, and Alan N. Sussman. “Considering the Definition of Addiction.” *International Journal of Environmental Research and Public Health*, vol. 8, no. 10, 20 Oct. 2011, pp. 4025–38, https://doi.org/10.3390/ijerph8104025.
4. Tyagi, Akanksha, et al. “Food-Derived Opioid Peptides in Human Health: A Review.” *International Journal of Molecular Sciences*, vol. 21, no. 22, 21 Nov. 2020, p. 8825, https://doi.org/10.3390/ijms21228825.
5. Thomas, Elizabeth A., et al. “Eating-Related Behaviors and Appetite during Energy Imbalance in Obese-Prone

https://doi.org/10.59720/22-127

and Obese-Resistant Individuals." *Appetite*, vol. 65, 10 Feb. 2013, pp. 96–102, https://doi.org/10.1016/j.appet.2013.01.015.

6. Blanco, E., et al. "Eating in the Absence of Hunger and Obesity among Adolescents in Santiago, Chile." *Journal of Community Health*, vol. 44, no. 5, Oct. 2019, pp. 874–80, https://doi.org/10.1007/s10900-018-00608-3.
7. Tallarida, Christopher S., et al. "Ethanol and Cocaine: Environmental Place Conditioning, Stereotypy, and Synergism in Planarians." *Alcohol*, vol. 48, no. 6, Sept. 2014, pp. 579–86, https://doi.org/10.1016/j.alcohol.2014.07.006.
8. Rawls, Scott M., et al. "Nicotine Behavioral Pharmacology: Clues from Planarians." *Drug and Alcohol Dependence*, vol. 118, no. 2–3, 1 Nov. 2011, pp. 274–79, https://doi.org/10.1016/j.drugalcdep.2011.04.001.
9. Dziedowiec, Emily, et al. "Mu Opioid Receptor Agonist DAMGO Produces Place Conditioning, Abstinence-Induced Withdrawal, and Naltrexone-Dependent Locomotor Activation in Planarians." *Neuroscience*, vol. 386, 27 June 2018, pp. 214-22, https://doi.org/10.1016/j.neuroscience.2018.06.029.
10. Nishimura, K., et al. "Analysis of Motor Function Modulated by Cholinergic Neurons in Planarian Dugesia Japonica." *Neuroscience*, vol. 168, no. 1, 16 June 2010, pp. 18–30, https://doi.org/10.1016/j.neuroscience.2010.03.038.
11. Kusayama, Taichi, and Shigeru Watanabe. "Reinforcing Effects of Methamphetamine in Planarians." *NeuroReport*, vol. 11, no. 11, 3 Aug. 2000, pp. 2511–13, https://doi.org/10.1097/00001756-200008030-00033.
12. Kosten, Thomas, and Tony George. "The Neurobiology of Opioid Dependence: Implications for Treatment." *Science & Practice Perspectives*, vol. 1, no. 1, July 2002, pp. 13–20, https://doi.org/10.1151/spp021113.
13. Zewde, Ashenafi Mebratu, et al. "PLDT (Planarian Light/Dark Test): An Invertebrate Assay to Quantify Defensive Responding and Study Anxiety-like Effects." *Journal of Neuroscience Methods*, vol. 293, 16 Oct. 2017, pp. 284–88, https://doi.org/10.1016/j.jneumeth.2017.10.010.
14. Spiga, Ralph, et al. "Effects of Nicotine on Methadone Self-Administration in Humans." *Drug and Alcohol Dependence*, vol. 50, no. 2, 1 Apr. 1998, pp. 157–65, https://doi.org/10.1016/s0376-8716(98)00020-9.
15. Dimy, Fluyau, and Charlton E Thomas. "Drug Addiction." *StatPearls*, StatPearls Publishing, 29 August 2022.
16. Raffa, Robert B. "Physical Dependence and Withdrawal in Planarians." *Planaria: A Model for Drug Action and Abuse*, CRC Press, 2019, pp. 67–73.
17. Ugur, Muzeyyen, et al. "Chronic Nicotine-Induced Changes in Gene Expression of Delta and Kappa-Opioid Receptors and Their Endogenous Ligands in the Mesocorticolimbic System of the Rat." *Synapse*, vol. 71, no. 9, Sept. 2017, https://doi.org/10.1002/syn.21985.
18. Ansari, Saleem, et al. "Adult Obesity Complications: Challenges and Clinical Impact." *Therapeutic Advances in Endocrinology and Metabolism*, vol. 11, 22 June 2020, https://doi.org/10.1177/2042018820934955.
19. Carter, Adrian, et al. "The Neurobiology of 'Food Addiction' and Its Implications for Obesity Treatment and Policy." *Annual Review of Nutrition*, vol. 36, 17 July 2016, pp. 105–128, https://doi.org/10.1146/annurev-nutr-071715-050909.